Adverse Challenges in the Perinatal Period May Alter Nociceptive Sensitivity in Later Life

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DECLARATION

I hereby declare that the composition of this thesis and the work presented are entirely my own, with the exception of ATF-3 immunohistochemisty, which was carried out by Andrew Allchorne and is included with his permission. Some of the data included in this thesis have been published as a poster communication and are included in the appendix.

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ABSTRACT

Chronic inflammatory and neuropathic pain states are poorly understood, and currently inadequately treated. Clinically, the symptoms of such pain states include allodynia (interpretation of innocuous stimuli as noxious), hyperalgesia (increased sensitivity to noxious stimuli) and spontaneous (non-evoked) pain. Additionally, chronic pain states are often associated with affective disorders such as anxiety and depression which can further reduce the individual's quality of life. It is highly likely that neuropathic pain could occur in combination with chronic inflammation, for example as a result of post-surgical infection. When such injuries occur in early life, during the continuing development of the nervous system, it is possible that long-term adverse changes in sensory processing may occur.

To investigate this, we have developed a rodent model of chronic pain with both a neuropathic and inflammatory component, designed to investigate the consequence of these to injuries coinciding. Furthermore, we are also investigating the effect of gestational stress, which has been shown to alter the stress responsiveness of the offspring and may also affect pain processing. To study the effect that prenatal stress may have on pain processing, we have utilised the rodent resident/intruder paradigm as a model of social stress to determine the outcomes of the combination of these adverse perinatal events.

We find that a combined inflammatory and neuropathic injury in the adult rat increases sensitivity to both mechanical and thermal stimuli and also increases spontaneous pain, when compared to inflammation or nerve injury alone. We show that neuropathic pain can be induced in neonatal (P8) rats; however there is little response to inflammation at P8 and a combination of these two injuries does not have the additive effect on sensitivity that occurs in the adult. Upon recovery from neonatal nerve injury, we find that a subsequent noxious challenge (formalin) alters nocifensive behaviour, when compared to the formalin response of naïve (no prior injury) animals, indicating long-lasting changes to nociceptive processing. Interestingly, when nerve injury is carried out in adult animals, nocifensive behaviour in response to formalin is not altered compared to naïve controls. Calcium entry through the NMDA receptor and subsequent CaM Kinase IIα activation has

been implicated as a crucial factor in long term potentiation (LTP) and the maintenance of sensitised states. In adult models of chronic pain, which may involve LTP mechanisms, we have shown an increased association of CaM Kinase IIα with NR2A/B spinal immunoprecipitates ipsilateral to injury. Furthermore, a different mechanism may be involved in neonatal pain states, as we have shown that spinal CaM Kinase IIα expression increases with development and is present at very low levels at the time of surgery in our pain models. Additionally, a number of other proteins associated with the NMDA receptor complex are developmentally regulated, and their involvement in the initiation and maintenance of chronic pain is likely to differ between the adult and the neonate.

We further show that exposure to prenatal stress does not alter the thresholds to mechanical stimuli in adult or early life pain models, however the combination of prenatal stress and postnatal injury results in an enhanced response to formalin in later life, indicating that programming of stress and/or pain pathways has occurred as a result of these early life events.

In addition to the development of a novel model of chronic pain, this study highlights the long-term impact that adverse perinatal events can have on offspring.

ABBREVIATIONS

11β-HSD2	11β-hydroxysteroid dehydrogenase 2
5-HT	Serotonin
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	Analysis of variance
ASIC	Acid-sensitive ion channel
ATF-3	Activating transcription factor 3
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BDNF	Brain derived neurotrophic factor
Ca^{2+}	Calcium ions
CaMKII	Calcium/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
Ca_v	Voltage-gated calcium channel
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
CGRP	Calcitonin-gene related peptide
Cl^-	Chloride ions
CNS	Central nervous system
CRH	Corticotropin releasing hormone
DRG	Dorsal root ganglia
DRt	Dorsal reticular nucleus
EDTA	Ethylenediaminetetraacetic acid
EP2	Prostaglandin E receptor
EPM	Elevated plus-maze
ERK	Extracellular signal-regulated kinase
GABA	Gamma-amino-butyric acid
GDNF	Glial-derived neurotrophic factor
Glu	Glutamate
GluR	Glutamate receptor subunit
GlyR	Glycine receptor

GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HPA	Hypothalamo-pituitary-adrenal
IASP	International Association for the Study of Pain
IB4	Isolectin B4
IL	Interleukin
JNK	c-Jun N-terminal kinase
K^+	Potassium ions
KA	Kainate receptor subunit
KCC2	Potassium-chloride co transporter
K_v	Voltage-gated potassium channel
LT	Low-threshold
LTD	Long-term depression
LTP	Long-term potentiation
MAGUKS	Membrane-associated guanylate kinases
MAPK	Mitogen-activated protein kinase
Mg^{2+}	Magnesium
mGluR	Metabotropic glutamate receptor
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
Na^+	Sodium ions
Na_v	Voltage-gated sodium channel
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NGF	Nerve growth factor
NK1	Neurokinin 1
NMDA	N-methyl-D-asparate
NR	NMDA receptor subunit
NS	Nociceptive specific
NSAID	Non-steroidal anti-inflammatory
ORL-1	Opioid receptor-like 1
P2X	Purinergic receptor subtype X channel
PAG	Periaqueductal grey

PB	Parabrachial area
pERK	phospho-ERK
PET	Positron emission tomography
PG	Prostaglandin
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PP	Protein phosphatase
PSD	Postsynaptic density
PVN	Paraventricular nucleus
PWL	Paw withdrawal latency
PWT	Paw withdrawal threshold
(R)-CPP	3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid
RIA	Radioimmunoassay
RVM	Rostral ventromedial medulla
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFL	Spontaneous foot-lifting
SP	Substance P
STT	Spinothalamic tract
TARP	Transmembrane AMPA receptor regulatory protein
TNF	Tissue necrosis factor
Trk	Tyrosine kinase
TRP	Transient receptor potential
TTX	Tetrodotoxin
WDR	Wide-dynamic range

CHAPTER 1: INTRODUCTION

1.1 Pain

"An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. The inability to communicate verbally does not negate the possibility that an individual is experiencing pain and is in need of appropriate pain-relieving treatment. Pain is always subjective. Each individual learns the application of the word through experiences related to injury in early life." (Merskey and Bogduk, 1994)

This definition of pain is important as it highlights the need to consider animals and human infants who cannot describe their pain or seek out appropriate treatment or relief. Many invasive procedures are carried out on human preterm infants, which may have adverse consequences in later life. It is therefore important that we study the consequences of potentially painful procedures in early life, in animals and humans, with a view to avoid pain and protect them from potentially adverse long-term changes to sensory processing.

It is important to differentiate between the terms "pain" and "nociception", as both will be used throughout this thesis. Pain encompasses both an unpleasant emotional or physical experience and involves higher processing and an awareness of the stimulus, whereas nociception involves only the neural detection of a noxious stimulus, which may then cause pain, together with any reflex motor responses.

Under normal, non-pathological conditions, pain results from activation of high threshold Aδ and C-fibres to warn of potential tissue damage. Chronic pain states (those persisting following recovery from initial injury) are a result of pathophysiological changes within the nervous system which can occur following nerve damage (neuropathic pain) or chronic inflammation and are currently difficult to treat (Scholz and Woolf, 2002). Broadly speaking, chronic pain is manifest as hyperalgesia (increased sensitivity to noxious stimuli), allodynia (interpretation of innocuous stimuli as noxious), and also dysethesias such as shooting pains, burning or itching which may be spontaneous or evoked. The current lack of effective treatments for chronic pain states and an estimated prevalence of 6-8% of the European population poses a major socio-economic problem. This is due to the high costs of treatments, which often repeatedly fail and also a loss of productivity with

patients unable to work because of chronic pain and/or its associated comorbidities (see 1.2.5).

1.2 Chronic Pain

1.2.1 Inflammation and chronic inflammatory pain

As with all types of acute pain, acute inflammation is an important physiological response that protects an organism from potential tissue damage. Inflammatory pain occurs when sensory nerve endings in the inflamed area are exposed to inflammatory mediators such as histamine and bradykinin (see 1.4.3). Once the initial stimulus or infection has been cleared, acute inflammation and the associated pain will subside. In cases of chronic inflammation the initial stimulus is not cleared quickly and the response continues. This can result in persistent activation of sensory neurons due to their increased excitability in the presence of inflammatory mediators, even in response to innocuous stimuli. In addition to this primary hyperalgesia, secondary hyperalgesia may also occur due to central sensitisation as a result of the persistent afferent input (see 1.8).

The innate immune system is responsible for defending the body in a non-specific manner, by recruiting immune mediators such as cytokines and initiating the complement cascade at the site of inflammation/infection. Timing of the maturation of the immune system is species specific, in offspring from precocious species (e.g. humans, pigs) most immune system development occurs *in utero* whereas in offspring from non-precocious species (rats and mice), immune development occurs late in gestation/early postnatally (Merlot, 2008). The cellular component of the immune response consists of mast-cells and macrophages whilst the soluble component is the complement cascade, both of these components of the inflammatory response have been implicated in chronic pain (Twining et al., 2005) and their developmental profile is important to consider with respect to the differences between adult and neonatal inflammatory pain.

1.2.2 Neuropathic pain

Neuropathic pain may arise as a result of damage to peripheral nerves or to the central nervous system. Such damage can occur in response to various injuries such as compression, stretch or transection of the nerve which may occur accidentally, as a result of major surgery or as a result of diseases such as diabetes (diabetic neuropathy), viral infection, alcoholism or stroke (Sadosky et al., 2008). Traditional analgesics such as non-steroidal anti-inflammatory drugs (NSAIDs) or opiates are thought to be less effective in treating neuropathic pain compared to nociceptive pain, and current treatments such as tricyclic antidepressants or anticonvulsant drugs vary in efficacy and have undesirable side-effects as well as unclear mechanisms of action (Finnerup et al., 2005; Dworkin et al., 2007).

1.2.3 Chronic pain and affective disorders

Chronic pain conditions are often accompanied by affective disorders such as depression and anxiety (Greden, 2009; Wiech and Tracey, 2009). Higher processing of noxious stimuli allows integration of these signals with memory and may result in recall of adverse experiences which can result in their association with negative mood, thus enhancing the affective nature of a given stimulus. The affective-emotive components of pain pathways are considered to be conferred by the spinoparabrachial pathway, as well as by ascending dorsal horn pathways connecting directly to limbic areas known to be involved in emotion and affect, such as the hypothalamus (spinohypothalamic) and amygdala (spinopontoamygdaloid) (Price, 2000). In addition to the difficulty in treating the underlying chronic pain state, these associated comorbidities further reduce the sufferers' quality of life. Limbic disturbances, resulting in depression and anxiety, associated with chronic pain are well documented (Blackburn-Munro and Blackburn-Munro, 2001; Ulrich-Lai et al., 2006). Sleep deprivation is another common problem in chronic pain patients and as well as adding to a depressed state, there is also evidence that it can exacerbate symptoms by reducing pain thresholds, making it more difficult to treat patients (Moldofsky et al., 1975; Smith et al., 2000; Nicholson and Verma, 2004).

1.2.4 Neuropathic versus inflammatory pain

In clinical situations, nerve injury may often be accompanied by some degree of acute or chronic inflammation, for example as a result of post surgical infection. Here, the inflammatory mediators responsible for chronic inflammation may potentiate the mechanisms involved in the development and maintenance of neuropathic pain. It has been suggested that the pathways involved in mediating pain associated with inflammation are quite different from those in neuropathic pain states and that the latter have a stronger affective component (Apkarian et al., 2008). It is therefore interesting to speculate on the outcome of a combination of these two types of injury and the potential synergistic effect this may have.

The molecular and physical changes involved in converting sensory neurons from a normal to a sensitised state differ between inflammatory and neuropathic conditions. One of the major mechanisms responsible and common to both conditions is the phosphorylation of receptors and ion channels in primary sensory neurons and in second order dorsal horn neurons that contribute to central sensitisation, discussed in detail below (1.8). Two important neurotransmitters differentially expressed in primary sensory neurons in inflammatory versus neuropathic pain states are substance P (SP) and calcitonin gene-related peptide (CGRP). These neuropeptides are released from the central terminals of primary sensory neurons to facilitate transmission in the dorsal horn and also from the peripheral terminals to exert local actions (see 1.7.1). SP and CGRP are upregulated in primary sensory neurons following inflammation (Smith et al., 1992) and can be released from the peripheral terminals to further increase the number of mast cells and macrophages (via vasodilation), adding to the "inflammatory soup" (Costigan and Woolf, 2000), which contains mediators responsible for peripheral sensitisation (see Figure 1.3). Following peripheral axotomy (Hokfelt et al., 1994) and chronic constriction injury (CCI) (Nahin et al., 1994) SP and CGRP mRNA levels are *downregulated* in primary sensory neurons and interestingly, the maximal decrease in SP mRNA has been shown to occur between 7-14 days which is around the time of peak sensitisation (Nahin et al., 1994), and is possibly due to C-fibre cell death as well as changes in gene expression profiles. Following nerve injury there is some evidence to suggest

that a switch occurs which converts some Aβ and Aδ fibres into a C-fibre-like phenotype (see 1.3.1), as they subsequently begin to express SP and CGRP, amongst other factors usually expressed only by small, unmyelinated C-fibres (Miki et al., 1998; Malcangio et al., 2000). In addition to this phenotypic switch, there is some evidence to suggest that central terminals of A-fibres may begin to sprout into the superficial lamina of the dorsal horn, an area which usually receives only nociceptive input from C-fibres (Woolf et al., 1992), although the validity and reliability of the tracing techniques used in these studies has been questioned (Tong et al., 1999; Hughes et al., 2003). Central sprouting of myelinated afferents might suggest that SP and CGRP would be released onto dorsal horn neurons in lamina II following activation of the large, myelinated fibres, which are activated by innocuous stimuli such as light touch or brushing of the skin. Therefore, innocuous stimuli may be perceived as noxious, thus contributing to the tactile/mechanical allodynia phenotype observed in pain clinics and in experimental pain models. However, conflicting studies (Hughes et al., 2007) find that there is no detectable increase in SP release from Aβ fibres following nerve injury. The authors suggest that discrepancies between studies on the involvement of SP from Aβ fibres in allodynia can be partially explained by the two different forms of allodynia that exist. Static allodynia is pain in response to light touch or pressure, which is mediated by TRPV1-positive (transient receptor potential vanilloid receptor 1, see 1.4.1) small to medium diameter C and Aδ fibres, whereas dynamic allodynia occurs in response to brushing the skin (Ochoa and Yarnitsky, 1993), which is mediated by TRPV1-negative medium to large Aδ and Aβ (Yamamoto et al., 2008). Studies that report analgesia in response to antagonists of the SP receptor, NK_1 (neurokinin 1) and therefore indicate SP involvement, have used von Frey filaments to measure allodynia and are therefore measuring static allodynia, which is mediated by C and Aδ fibres (Cahill and Coderre, 2002). Also, it is important to realise that although there may be expression changes in the DRG, this may not necessarily translate to changes in SP release from central terminals.

There is some evidence to suggest that sodium channels are also altered differently in inflammatory versus neuropathic pain states; specifically, the voltage-gated sodium channel, $Na_v1.8$ has been implicated in chronic pain conditions. A recent study

utilising conditional knockouts for this channel suggests that $Na_v1.8$ is important in the generation of pain behaviour following inflammation, as both the formalin response, and thermal hyperalgesia associated with injection of complete Freund's adjuvant are attenuated, whereas neuropathic pain is unaffected (Abrahamsen et al., 2008). Other groups have shown that $Na_v1.8$ is significantly downregulated following nerve injury, which is considered to be due to its replacement by $Na_v1.3$ (Dib-Hajj et al., 1999) (See 1.4.4).

The changes discussed above and the many other changes at the peripheral level in inflammatory and neuropathic pain states all result in increased activity of primary sensory neurons (Millan, 1999; Zimmermann, 2001). The increased firing of these neurons will in turn increase excitatory synaptic transmission between dorsal horn neurons and activate postsynaptic glutamate receptors to generate a cascade of postsynaptic pathways which lead to phosphorylation of the AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and NMDA (N-methyl-D-asparate) glutamate receptors, lowering their activation thresholds and therefore increasing excitability in the dorsal horn (central sensitisation) (Raymond et al., 1994; Lee, 2006).

1.2.5 Animal models of chronic pain

The subjective nature of pain makes it very difficult to achieve reliable results from studies on human subjects. Furthermore, it is impossible, both ethically and practically, to accurately investigate the mechanisms responsible for the broad range of symptoms associated with chronic pain in humans. For these reasons, animal models of chronic pain are widely used, although it is often difficult to directly translate experimental results to effective clinical treatments due to the differences in physiology between rodent and human subjects, and also the differences in the duration and symptoms associated with clinical neuropathic or inflammatory pain states, which can rarely be accurately encompassed by animal models. Nevertheless, there have been a number of important discoveries in the chronic pain field, which have utilised animal models to increase our understanding of these complex disorders. Some of the most widely used models of neuropathic pain are spinal or

peripheral nerve injuries, which include the chronic constriction injury (CCI), a loose ligation of the sciatic nerve to induce intraneural oedema and physical axonal damage (Bennett and Xie, 1988), spinal nerve (L5 and 6) ligation, (Kim and Chung, 1992), partial sciatic nerve ligation, (Seltzer et al., 1990) and more recently the spared nerve injury, in which the tibial and common peroneal nerve are ligated and the sural branch of the sciatic nerve left intact (Decosterd and Woolf, 2000). In addition to these nerve injuries, animal models have also been produced to mimic specific disease-induced neuropathies, such as diabetic neuropathy (Malcangio and Tomlinson, 1998) and postherpetic neuralgia (Fleetwood-Walker et al., 1999).

In addition to neuropathies, chronic inflammatory pain is also problematic clinically. A number of animal models of inflammatory pain exist which vary in duration and mechanism. The formalin test is a well-defined acute (usually up to 60 mins) nociceptive test (Dubuisson and Dennis, 1977), which is now know to directly activate TRPA1 receptors (McNamara et al., 2007) thought to be responsible for the first wave of pain behaviours observed upon injection into the hindpaw. The second phase of the formalin response is longer in duration and thought to be due to ongoing inflammatory input as well as central sensitisation induced by activation of nociceptors during the first phase (McNamara et al., 2007). The formalin test is a useful test to investigate central sensitisation and changes in central processing which may have occurred as a result of a prior injury. Capsaicin is a similar noxious inflammatory agent that directly activates TRPV1 receptors (Caterina et al., 1997) to produce an acute behavioural response. Longer-lasting inflammatory agents include immune system activators such as carrageenan and complete Freund's adjuvant (CFA). These agents are often used to model arthritis by injecting into the knee or ankle joints but are also use for studying chronic inflammation and inflammatory processes following an intraplantar injection in the hindpaw.

These animal models all differ in the duration and extent of the nociceptive behaviours observed and it is important to select the most appropriate model for a particular study. Although many peripheral neuropathies have an inflammatory component at the site of injury, a more direct combined model of neuropathic and inflammatory pain has not yet been investigated and may in fact, be a more realistic model of the injuries associated with certain animal husbandry practices such as

those experienced by piglets following tail-docking and also by human adults or neonates experiencing post-surgical infection. Tail-docking is currently carried out routinely in new-born piglets and results in obvious damage to the peripheral nerves and wounds are often prone to infection and inflammation. Clinically similar coincident nerve damage and inflammation is likely to occur following surgery in both adult humans and also pre-term infants. A synergistic effect of these two injuries is a likely outcome and the resulting pain experienced acutely and in the long-term warrants further investigation, not least in younger subjects where development of the nervous system is incomplete at the time of injury and could potentially be adversely affected long-term.

1.2.6 Measures of pain: Evoked versus spontaneous behaviour

Due to the emotional and subjective nature of pain, quantifying a painful experience accurately in laboratory animals is extremely difficult, if not impossible. Therefore most animal models of chronic pain are tested using spinally-mediated reflex responses to noxious stimuli such as von Frey filaments, to measure mechanical allodynia or the Hargreaves' plantar test to measure thermal hyperalgesia. These tests measure stimulus-evoked responses to noxious stimuli, which we can refer to as nociceptive behaviour, as only the sensory pathways at the peripheral and spinal level are considered due to the reflex nature of the response (see definition 1.1). However, spinally mediated reflex withdrawal responses do not require higher processing at the level of the cortex and therefore the affective-emotive nature of pain is not considered in such tests. Furthermore, there have been a number of reports which show differential responses to reflex-based measures when compared to operant and stimulus-response based assessments, which require cortical processing (Vierck et al., 2002; Vierck, Jr. et al., 2004; Vierck et al., 2008).

Data from clinical studies show that spontaneous pain makes up a large part of the symptoms of chronic pain patients (Backonja and Stacey, 2004). Much of the current research using animal models of chronic pain focuses on evoked responses, when in fact, the prevalence of symptoms in human patients shows that perhaps there should be more focus on spontaneous nociceptive behaviour. There are a number of ways to

measure spontaneous pain, and recently, conditioned place preference tests have been used to show ongoing pain following peripheral nerve injury in rats. In these tests, injured rats will actively seek out chambers with which they have been pre-conditioned to associate with pain relief (King et al., 2009). Additionally, preclinical trials utilising stimulus-evoked measures of hypersensitivity, do not always translate to drugs that are effective in human patients. For these reasons, it may be helpful if novel preclinical drugs were also tested for their effectiveness in alleviating spontaneous pain, in ways which consider whole animal behaviour and allow for assessments which can be more directly translated to the human experience of pain.

1.3 Pain Pathways: Organisation of the somatosensory system

1.3.1 Primary sensory neurons

Primary sensory neurons have their cell bodies in the dorsal root ganglia (DRG) or trigeminal ganglia. From each cell body a single axon arises, which then bifurcates to give rise to a distal branch which extends out to the periphery and a proximal branch which terminates at second order neurons within the dorsal horn of the spinal cord to relay sensory information to central synapses. The peripheral terminals of primary sensory neurons are able to detect and transduce a wide range of sensory modalities which can be of a thermal, chemical or mechanical nature. To allow for such wide variety of potential stimuli, many primary sensory neurons are polymodal and their afferents can be divided into three main types depending on size and conduction velocity. Aβ fibres are large-diameter, myelinated axons with a high conduction velocity and respond to tactile, non-nociceptive, stimuli but may be involved in pathological pain states in contributing to allodynia (Woolf and Doubell, 1994). Aδ fibres are medium-diameter and thinly myelinated with a conduction velocity in the range of 2.2-8 ms^{-1} and C-fibres are small-diameter, unmyelinated axons and conduct slowly at <2.2 ms^{-1} in the rat (Harper and Lawson, 1985). It is the Aδ and C-fibre terminals that respond to noxious stimuli and make up the majority of nociceptors, although they are also responsible for innocuous temperature perception and itch (Schmelz et al., 1997; Schepers and Ringkamp, 2009). Adult C-fibres can be further

sub-divided according to their protein expression profiles. The peptidergic C-fibre population expresses the TrkA (tyrosine kinase A) receptor for nerve growth factor (NGF), and the peptide neurotransmitters SP and CGRP and BDNF (brain-derived neurotropic factor) which binds to TrkB receptors on dorsal horn neurons (Malcangio and Lessmann, 2003). The non-peptidergic population can be selectively labelled by isolectin B4 (IB4) and expresses the Ret receptor for the growth factor glial-derived neurotrophic factor (GDNF) (Bennett et al., 1998). In the perinatal period this classification of C-fibres is not so straight-forward, as developing neurons require NGF for survival and therefore a higher proportion of C-fibres will express its receptor, TrkA, which decreases to adult levels by postnatal day 14, whilst IB4 binding increases (Bennett et al., 1996).

Nociceptive mechanical and thermal information is transduced primarily by the high-threshold Aδ and C-fibres upon activation of one or more of the many types of receptors (see 1.4) located on their terminals. The majority of the sensory receptors located on the terminals of primary sensory neurons are ionotropic cation channels (Lee et al., 2005), which upon activation lead to depolarisation of the neuron and the generation of an action potential. This is conveyed along the axon to the central terminal within a specific area of the dorsal horn, which depends on its peripheral terminal location and also the afferent type.

1.3.2 Central organisation within the dorsal horn

The dorsal horn of the spinal cord is divided into 6 laminae that receive and process sensory information (Figure 1.1.) (Rexed, 1952; Molander et al., 1984). The order in which primary sensory neurons enter the dorsal horn is highly organised, with non-nociceptive Aβ fibres entering lamina III-VI and smaller diameter Aδ and C-fibres primarily terminating in lamina I and II (Lamina II is usually further subdivided into inner, IIi, and outer, IIo, layers), which make up the superficial dorsal horn where a proportion of the cells are nociceptive specific (NS) and respond only to noxious stimuli (Christensen and Perl, 1970; Dubner and Bennett, 1983; Mason, 2007). The majority of Aδ fibres terminate in lamina I, with very few terminating in lamina II, whilst C-fibres terminate within particular regions

of the superficial dorsal horn depending on their subclass. The non-peptidergic, IB4-positive C-fibres terminate almost exclusively in lamina II (Kitchener et al., 1993) where they synapse with interneurons, which make up most of the neuronal population of lamina II. These then convey information to lamina V projection neurons to transmit signals to the brain mainly via the spinothalamic tract (see 1.3.4 & Figure 1.1.). The SP-containing peptidergic population of C-fibres terminate in lamina I where the NK_1 receptor is found, on approximately 80% (Marshall et al., 1996) of the projection neurons in this lamina, which transfer nociceptive signals to the brainstem and thalamus (Todd et al., 2002; Spike et al., 2003; Braz et al., 2005).

1.3.3 Dorsal horn processing and output: Projection neurons, interneurons and glia

The two main cell types within the dorsal horn of the spinal cord receiving peripheral input are interneurons (the majority) and projection neurons. Projection neurons are mainly located in lamina I and lamina V and project to the thalamus or to the brainstem (Gauriau and Bernard, 2002; Todd, 2002). The cells responsible for the output from the dorsal horn can be categorized into three types, depending on the information they respond to. Low-threshold (LT) neurons are only excited by touch (Aβ fibre input), or innocuous temperature (Aδ/C-fibres input). LT neurons are mainly found in lamina I, III and IV and have small receptive fields, which would suggest that they are able to encode stimulus location (Price and Dubner, 1977; Willis and Coggeshall, 1991; Schepers and Ringkamp, 2009). As mentioned previously, there are a substantial number of cells within the superficial dorsal horn (laminae I and II) that are nociceptive-specific (NS), so will only fire action potentials in response to painful stimuli. The third type of neuron found in the dorsal horn, predominantly in lamina V, are the wide dynamic range (WDR) neurons which, as the name suggests, will respond to a variety of stimuli, noxious or innocuous (Price and Dubner, 1977; Dubner and Bennett, 1983). WDR neurons respond to all three types of primary afferents and fire in a graded manner, according to the intensity of the stimulus. These WDR cells are able to exhibit a short-lasting form of synaptic plasticity known as wind-up, which is elicited by repeated low

frequency stimulation, particularly of C-fibre intensity (Mendell, 1966), and may be involved in initiating longer-lasting forms of synaptic plasticity such as central sensitisation and long term potentiation (1.8.2), which are implicated mechanisms in chronic pain states (Ji et al., 2003).

Excitatory (largely glutamatergic) and inhibitory (largely GABAergic) interneurons act on all three types of the above mentioned cells to alter the output from the dorsal horn. Interneurons are found throughout the dorsal horn laminae, although they make up the majority of the cells in lamina II. The processes of most interneurons are restricted to the segment of spinal cord containing their cell body, and these are known as local circuit neurons. Other interneurons have longer-range axons which extend to different spinal segments. Through activation from primary afferents or descending pathways (see 1.3.6) inhibitory and excitatory interneurons are able to modulate dorsal horn output by connecting with other interneurons, directly contacting the presynaptic terminals of primary afferents, and by directly contacting projection neurons (Willis and Coggeshall, 1991; Hunt and Koltzenburg, 2005).

Glial cells are non-neuronal cells found within the nervous system and are divided into microglia and macroglia. Microglia are specialized macrophages which form the main immune defence in the CNS. Macroglia are mainly made up of astrocytes and oligodendrocytes in the CNS, and regulate the external chemical environment and provide nutrients to neurons and produce the myelin sheath, respectively, both of which facilitate efficient neurotransmission. Glial cells do not produce action potentials but are able to facilitate signalling in the their supportive roles and have also been shown to respond to and release neurotransmitters (such as glutamate and ATP) and a variety of neuromodulatory cytokines and therefore play an important role in nociceptive processing within the dorsal horn (Barres, 1991; Scholz and Woolf, 2007). It is only relatively recently that the role of glial cells in nociceptive processing has become more widely investigated, particularly following injury (see 1.6) (Meller et al., 1994; Scholz and Woolf, 2007; Vichaya et al., 2009) in both the development and maintenance of chronic pain states (Raghavendra et al., 2003; Hains and Waxman, 2006; Ji and Suter, 2007; Vichaya et al., 2009). The signalling mechanisms involved within these cells during persistent pain states are currently under investigation with the aim of identifying potential therapeutic targets.

1.3.4 Ascending pathways

Nociceptive and non-nociceptive somatosensory information is transferred rostrally from the dorsal horn to the brain via many different pathways (Figure 1.1.). The dorsal column-medial lemniscal pathway carries information about touch, vibration and proprioception and synapses in the medulla and thalamus before information reaches the cortex (Purves and Williams, 2001). The main pathway responsible for the transmission of pain signals is the spinothalamic tract (STT) which mostly originates from deep dorsal horn laminae and terminates in the ventroposterior and ventrobasal thalamus, and the spinoparabrachial pathway (also referred to as part of the spinoreticular tract) which originates primarily from the superficial dorsal horn and synapses in brainstem, midbrain and limbic areas before reaching the thalamus (Dubner and Bennett, 1983; Willis and Westlund, 1997; Hunt and Mantyh, 2001; D'Mello and Dickenson, 2008). The STT has previously been thought of as the most important pathway in pain signalling, particularly the fast discriminative properties of pain, due to the small receptive fields of the STT cells (Willis et al., 1974) and its direct contact with the thalamus (see below 1.3.5). Most of the input to this pathway arises from laminae I and V (Dubner and Bennett, 1983; Willis and Westlund, 1997), which both receive input from nociceptors (1.3.2 and 1.3.3), although neurons within the STT are responsive to both noxious and innocuous stimuli, with approximately 20% of the fibres being of the LT type (Dubner and Bennett, 1983). More recently focus has moved from the STT to the spinoparabrachial pathway. The spinoparabrachial pathway is mainly made up of NS neurons whilst a small proportion seem to be WDR neurons, with very few neurons responding exclusively to innocuous stimuli (Bester et al., 2000). Furthermore, this pathway mainly originates from the NK_1-positive cells in lamina I of the dorsal horn (McMahon and Wall, 1985; Bester et al., 2000), which are thought to signal the intensity of noxious stimuli (Doyle and Hunt, 1999). In agreement with this, the cells of the spinoparabrachial pathway have been shown to have increased responses to increasing stimuli intensity (Bester et al., 2000). Along with the limbic connections of the spinoparabrachial tract, these properties suggest that this pathway may contribute to conferring the affective dimensions of pain, particularly its intensity

and unpleasantness, and could potentially also have a role in associated comorbidities such as anxiety and depression. In addition to the spinoparabrachial tract there are also more direct pathways from the dorsal horn that contact the hypothalamus and amygdala (1.2.5). These are considered to arise from cells regulated by the non-peptidergic population of C-fibres (see 1.3.2) and may also contribute to the affective nature of some pain states (Burstein et al., 1987; Burstein and Potrebic, 1993; Willis and Westlund, 1997; Braz et al., 2005).

Figure 1.1

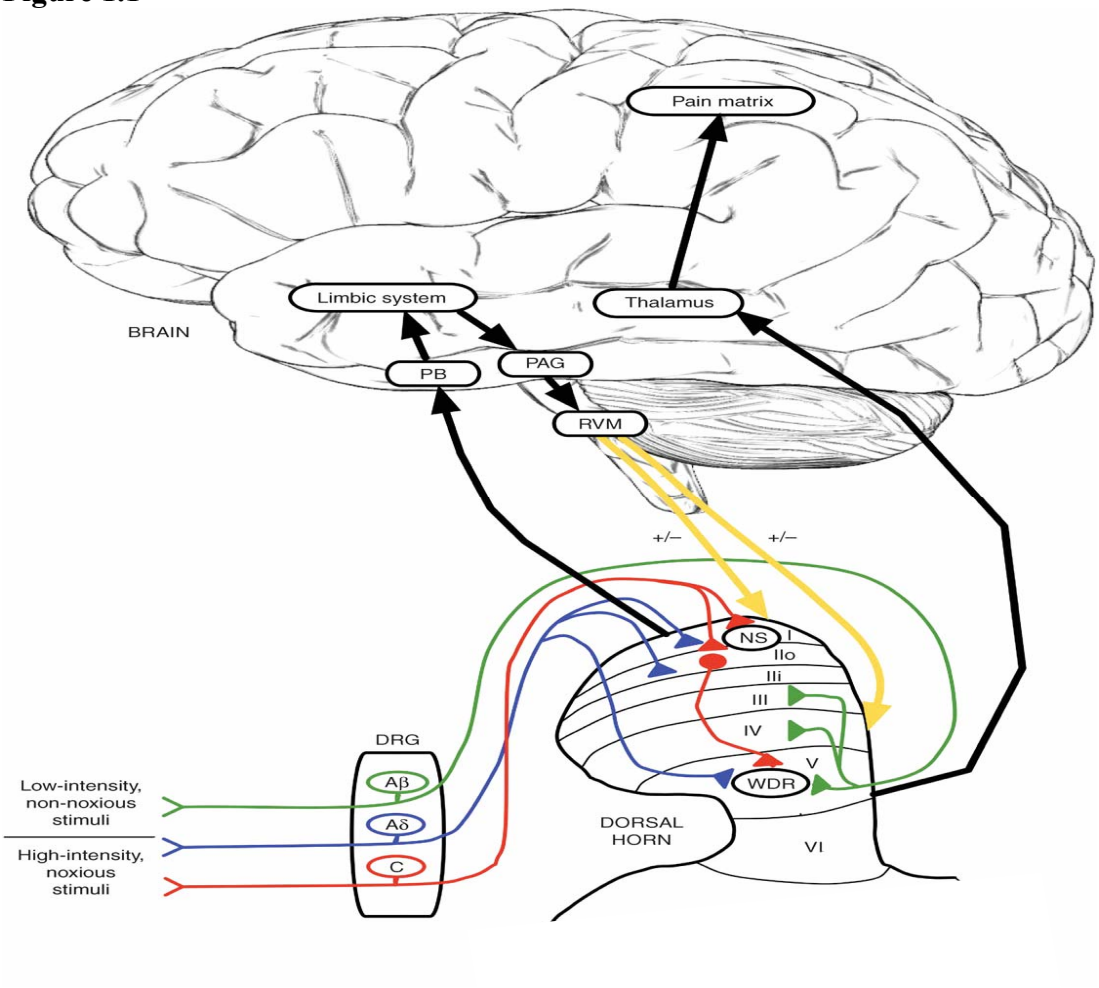


Figure 1.1. Diagram to illustrate the laminar organisation of dorsal horn input and its ascending (black) and descending pathways (yellow) to and from the brain and brainstem (D'Mello and Dickenson, 2008). Nociceptive information enters the dorsal horn through Aδ (blue) and C-fibres (red) which terminate in the superficial dorsal horn (lamina I and IIo) and in the deep dorsal horn (directly and indirectly) at lamina V where they contact wide-dynamic range (WDR) neurons. Nociceptive specific (NS) neurons are contacted by C-fibres in the superficial dorsal horn. Non-noxious information is relayed via Aβ fibres (green) which terminate in lamina III-V, where they also contact WDR neurons. Information is transferred rostrally from NS neurons in the superficial dorsal horn via the spinoparabrachial tract (black arrow) to the parabrachial area (PB) before reaching limbic areas and the thalamus. WDR in the deep dorsal horn ascend via the spinothalamic tract (black arrow) to reach the thalamus. Descending control from noradrenergic and serotonergic pathways originates in the periaqueductal grey (PAG) and rostral ventromedial medulla (RVM) (yellow arrows). For additional associated text see Sections 1.3 and 1.7.

Figure 1.2

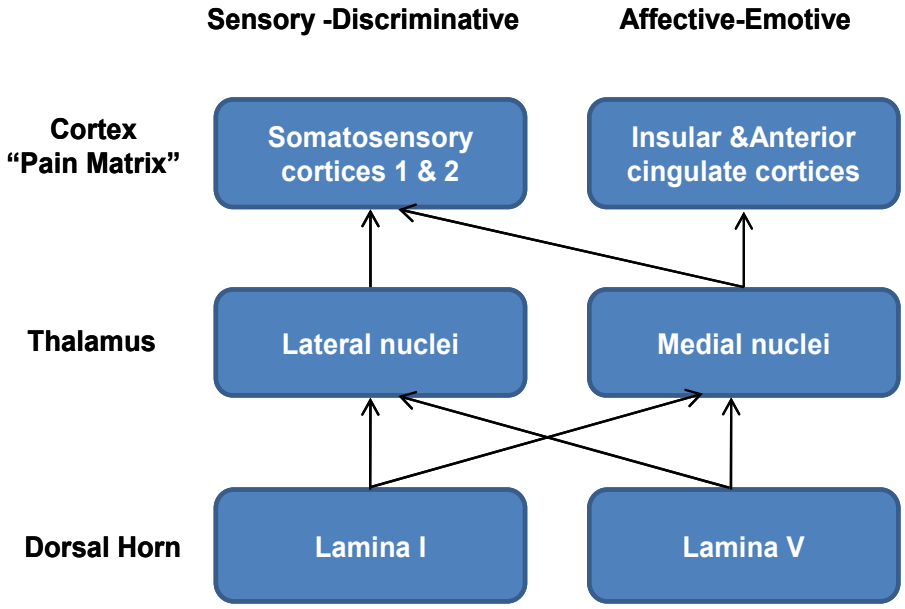


Figure 1.2. Simplified diagram of the cortical connections of the spinothalamic tract which originates in lamina I and V and connects in the lateral or medial nuclei of the thalamus before reaching cortical areas where the two major components of pain, sensory-discriminative and affective-emotive are transduced. (Ingvar, 1999)

1.3.5 Brain areas

Modern neuroimaging techniques such as PET (Positron Emission Tomography) and fMRI (functional Magnetic Resonance Imaging) have allowed non-invasive observation of the brain to view the cortical representation of pain. These imaging techniques show that multiple areas are activated in response to painful stimuli; furthermore, the areas associated with the different components of pain perception are beginning to be identified (Peyron et al., 2000). The ascending pathways from the spinal cord project to many brainstem and brain areas (see above 1.3.4 and Figure 1.1.), such as the parabrachial area and limbic areas such as the amygdala and hypothalamus, before reaching the thalamus which receives and processes all nociceptive information. From the thalamus, pain information is relayed to higher cortical areas, referred to as the "pain matrix" (Treede et al., 1999) which includes the primary and secondary somatosensory cortices (S1 and S2, respectively), and the

insular, anterior cingulate and prefrontal cortices. It is widely accepted that these brain areas represent the different components of the pain response, which can be broadly grouped into the sensory-discriminative component (location, intensity, type), and the affective-emotive component (anxiety, fear, anticipation) (Treede et al., 1999; Brooks and Tracey, 2005; D'Mello and Dickenson, 2008). The sensory-discriminative component is considered to be processed via the lateral nuclei of the thalamus and S1 and S2, and both the thalamus and somatosensory cortex have a somatotropic organisation of the receptive fields to convey location, making this component of pain similar to the other classical senses (see Figure 1.2). The affective nature of pain and its ability to evoke emotive responses is understood to be largely mediated by pathways through the medial thalamic nuclei, which have connections with the limbic brain and ascend to the insular and anterior cingulate cortex as well as S2 (Treede et al., 1999; Price, 2000). Of course this is a simplified view of the brain areas involved in pain processing, and there are many interconnecting pathways that integrate the various components of a painful experience to facilitate suitable physiological responses.

1.3.6 Descending pathways

As well as the ascending spinal pathways to the brain, there are also descending pathways from the brain and brainstem which synapse with targets within the dorsal horn (primary afferents, interneurons or projection neurons) to influence its output. Two of the main areas exerting descending control on the dorsal horn are the periaqueductal grey (PAG) and the rostral ventromedial medulla (RVM) in the brainstem (Figure 1.1 and section 1.7). These brainstem-spinal pathways were previously thought of as predominantly inhibitory, but they may also facilitate nociceptive transmission (Basbaum and Fields, 1984; D'Mello and Dickenson, 2008). The descending pathways from the brain to the spinal cord are complex. To briefly summarize, the PAG and amygdala have few direct projections to the dorsal horn but can modulate information from the thalamus and hypothalamus as well as from the "pain matrix" (see above 1.3.5) and they project to the dorsal horn via the RVM and other brainstem nuclei (Millan, 2002). These descending pathways are predominantly

serotonergic or noradrenergic and their effect is determined by the neuron contacted and also the receptor sub-type activated (Millan, 2002). Recent studies have also identified an oxytocinergic pathway projecting from the paraventricular nucleus (PVN) of the hypothalamus to the superficial dorsal horn where it is proposed to exert an inhibitory effect on nociceptive processing via indirect contact with GABAergic interneurons (Rojas-Piloni et al., 2007; Condes-Lara et al., 2007; Rojas-Piloni et al., 2008). This pathway may be important in mediating stress-induced analgesia as the PVN is part of the hypothalamo-pituitary-adrenal (HPA) axis which is activated in response to stress, and oxytocin neurons in the PVN are activated by a variety of stressors (see 1.12).

Descending pathways from the brain to the spinal cord also contact sympathetic neurons in the ventral horn and can modulate sympathetic outflow primarily via serotonergic and noradrenergic influences (Millan, 2002); a factor that becomes important in chronic pain states (see 1.3.7 and 1.7)

1.3.7 Sympathetic nervous system

The sympathetic nervous system is a branch of the autonomic nervous system (along with the parasympathetic nervous system) and is responsible for the "flight or fight" response, which is most active during times of stress or fear. Preganglionic spinal sympathetic neurons synapse with peripheral sympathetic neurons (postganglionic) through a series of ganglia. The principal neurotransmitters at sympathetic synapses are acetylcholine, which is released from preganglionic axons, and noradrenaline, released from postganglionic fibres, to act on α-adrenoreceptors in target tissues. Postganglionic neurons do not usually communicate with the afferent neurons within the periphery and are therefore not usually involved in the generation of pain. However, in cases of peripheral sensory nerve damage, sympathetic efferents can couple with primary afferents and may be involved in the maintenance and exacerbation of neuropathic pain states through noradrenergic signalling (McLachlan et al., 1993; Janig and Habler, 2000; Pertin et al., 2007).

1.4 Sensing Pain: Molecular mediators of peripheral signal detection and propagation

Cutaneous nociceptors can be divided into three main types, mechanoreceptors, thermoreceptors or chemoreceptors, depending on the stimuli they respond to. C-fibres are often broadly described as polymodal as many will respond to all three types of stimuli (Bessou and Perl, 1969). Aδ fibres respond to intense mechanical stimuli and can also respond to thermal stimuli, especially in sensitised conditions such as those that occur in response to nerve injury (Ji et al., 2007). Some nociceptive neurons, largely C-fibres, are also referred to as "silent", as they are not normally responsive to noxious stimuli and only function in pathological conditions (Schmidt et al., 1995). Centrally, synapses which contain NMDA receptors but lack AMPA receptors are postsynaptically "silent" at resting membrane potentials (Isaac et al., 1995) due to the voltage-dependant Mg^{2+} block of the NMDA receptor and inability of the postsynaptic neuron to be depolarised in the absence of AMPA receptors (see 1.5.3). Trafficking of AMPA receptors to the postsynaptic membrane and the subsequent activation of these previously silent synapses has been proposed as a potential mechanism for increased synaptic efficacy (Kessels and Malinow, 2009), a feature associated with central sensitisation in chronic pain states (see 1.5.1 and 1.8.3 for more detail). Nociceptors are able to respond to such a wide array of stimuli due to the broad range of receptors located at their terminals and the signal transduction mechanisms accompanying them. Most of these sensory receptors require the activation of ionotropic cation channels which allow an influx of cations and therefore depolarisation of the nerve when activated. There are also many metabotropic receptors that can facilitate transmission indirectly by modification and activation of ion channels (Lee et al., 2005).

1.4.1 Transient receptor potential (TRP) ion channels

TRP ion channels are the main receptors in the periphery responding to noxious heat (~>43ºC) and cold (~<15ºC), although they may also respond to mechanical and chemical stimuli. The primary molecular sensors of mechanical nociception remain

poorly defined, although the TRPA1 channel has been suggested to play a role sensing both noxious mechanical and chemical stimuli (Macpherson et al., 2007; Petrus et al., 2007). Six types of thermosensitive TRP channels have been identified on primary sensory neurons; these are TRPV1-4, and TRPM8 and TRPA1. TRPV1 and TRPV2 are activated by noxious heat with thresholds above 43 ºC (Caterina et al., 1997) and 52 ºC (Caterina et al., 1999) respectively. TRPV1 is also responsive to chemical stimuli such as capsaicin, endocannabinoids, acid, spider toxins (e.g. vanillotoxins) and it has also been reported to be potentiated by ethanol (Trevisani et al., 2002; Siemens et al., 2006; Levine and Alessandri-Haber, 2007). Furthermore, TRPV1 is also sensitised by many inflammatory mediators such as bradykinin, through phosphorylation-dependent mechanisms, to reduce its activation threshold temperature and contribute to the thermal hypersensitivity associated with inflammation (Sugiura et al., 2002; Wang and Woolf, 2005; Lee et al., 2005). TRPV3 and TRPV4 respond to innocuous warm temperatures (Levine and Alessandri-Haber, 2007) and so are not classed as thermal nociceptors, however, TRPV4 is responsive to chemical stimuli such as acid and is also implicated in mechanical and thermal nociception, from studies on mice lacking TRPV4 (Suzuki et al., 2003). Innocuous and noxious cold appear to be detected by TRPM8 and TRPA1, which are activated at temperatures below 25-28 ºC and 17 ºC respectively (Peier et al., 2002; Story et al., 2003). Although it is unclear whether TRPM8 responds to noxious cold (Fleetwood-Walker et al., 2007), it is also activated by menthol and icilin which have been shown to provide analgesia in response to nerve injury by mechanisms thought to involve inhibitory spinal metabotropic glutamate receptors (see 1.5.4) (Proudfoot et al., 2006). TRPA1 is also activated by many chemical stimuli including icilin, menthol and mustard oil and has recently been implicated in mediating the nocifensive response to formalin (Macpherson et al., 2007; McNamara et al., 2007)

1.4.2 Other ligand-gated ion channels involved in nociception

There are many channels found on peripheral afferents that respond to noxious stimuli and are implicated in nociception, along with the TRP channels. Such other

ion channels include the acid-sensitive ion channel family (ASIC) and the purinergic receptor subtype X channel family (P2X-). ASICs are activated by acid which may occur during local acidosis in cases of inflammation or infection, whereas P2X channels are activated by ATP (adenosine tripohosphate) which is released from damaged cells, following inflammation or mechanical tissue damage (Leffler et al., 2006; Inoue, 2007).

1.4.3 Metabotropic receptors

Activation of metabotropic receptors (G protein-coupled or tyrosine kinases) results in intracellular signal cascades which may ultimately modulate opening or closing of ion channels to affect transmission. In inflammatory pain states, following tissue damage for example, a number of factors (e.g. 5-HT, bradykinin, histamine, NGF, prostaglandins) are released from mast cells and macrophages and can act on metabotropic receptors on the primary sensory neuron to generate a series of intracellular events which can lead to ion channel activation and may sensitise the peripheral terminal (see 1.4.5)(Costigan and Woolf, 2000).

1.4.4 Voltage-gated ion channels

Sodium

Voltage-gated sodium channels (Na_v) open in response to a change in membrane potential and are responsible for the rapid depolarisation required for the generation of an action potential. Voltage-gated sodium channels exist in three states, which are: deactivated (closed), activated (open; by a change in voltage) and inactivated (closed; shortly after opening to allow for repolarisation). There are nine voltage-gated sodium channels expressed in humans and four of these have been implicated in nociception based on their distribution, expression profiles and functional properties. $Na_v1.7$, 1.8 and 1.9 are expressed preferentially in small diameter peripheral neurons whilst $Na_v1.3$ is expressed at high levels during fetal development and expression declines at birth but is re-expressed in DRG following nerve injury

(Waxman et al., 1994). These channels are also classified according to their sensitivity to the sodium channel blocker tetrodotoxin (TTX), $Na_v1.3$ and $Na_v1.7$ (amongst others) are sensitive to TTX (TTX-S) whilst $Na_v1.8$ and $Na_v1.9$ are resistant (TTX-R) (Krafte and Bannon, 2008). One of the major modifications to occur within the peripheral terminal in response to injury is the phosphorylation of $Na_v1.8$ by protein kinase A (PKA) and protein kinase C (PKC) (Gold, 1999) (see Figure 1.3.). This lowers the voltage activation threshold of the neuron, facilitating propagation of an action potential in response to the detection of noxious stimuli and so increases sensitivity in the area it innervates. This may be more important in the early stages of hypersensitivity as $Na_v1.8$ deficient mice show a delayed onset of thermal sensitivity following inflammation rather than a complete absence of it (Akopian et al., 1999). In contrast to these post-translational changes in $Na_v1.8$ sodium channels, a decrease in $Na_v1.8$ and Na_v 1.9 mRNA expression levels have been observed in DRG following nerve injury (Dib-Hajj et al., 1999). These particular types of sodium channel are expressed exclusively in primary sensory neurons and have slower activation kinetics than TTX-S types such as $Na_v1.3$ (Waxman et al., 1994; Dib-Hajj et al., 1999). The replacement of these TTX-R channels with the TTX-S, $Na_v1.3$, in primary sensory neurons will allow the neuron to fire repetitively due to faster activation kinetics as well as quicker recovery from the inactivation state (Cummins et al., 2001), furthermore this increased excitability is likely to be a contributing factor to the hyperalgesia and allodynia observed in neuropathies. There is little evidence from knockout studies for an involvement of $Na_v1.7$ and $Na_v1.9$ in neuropathic pain states, however they are implicated in inflammatory pain states (Nassar et al., 2004; Amaya et al., 2006) and gain of function mutations of the $Na_v1.7$ gene have been implicated in human pain disorders such as primary erythermalgia, (Drenth and Waxman, 2007) a condition where affected individuals experience a severe burning sensation in the extremities in response to heat or exertion.

Calcium

Intracellular calcium is involved in regulating a number of biochemical pathways and its concentration is therefore usually tightly controlled within the cell. Voltage-gated calcium channels (Ca_v) located in the CNS and periphery play vital roles in pain processing by allowing calcium to enter the cell in specific circumstances to alter membrane excitability, neurotransmitter release and gene expression. These channels can be broadly divided into two groups based on their activation thresholds, which are then further divided into subfamilies designated Ca_v1-3, based on the properties of the pore forming α_1 subunit that determines the functional properties of the channel. High threshold voltage-gated calcium channels include the L (Ca_v1), type and the N, R and P/Q (Ca_v2) types; low threshold voltage-gated calcium channels are the T (Ca_v3) type calcium channels (Gribkoff, 2006). The high threshold N type, $Ca_v2.2$ channel is highly expressed in nociceptive primary sensory neurons (Aδ and C-fibres) and has therefore been implicated as one of the most important voltage-dependent calcium channels in pain processing (Gribkoff, 2006). Furthermore, knockouts of the α subunit specific to this channel show reduced reflex responses to noxious mechanical and thermal stimuli and reduced pain behaviour in the second phase of the formalin response, indicating a role at the spinal level where this channel is abundant on presynaptic terminals (Kim et al., 2001). A number of drugs based on modification of calcium channel activity have been utilised to treat chronic pain, perhaps the most widely used is the antiepileptic drug, gabapentin. Gabapentin selectively binds to voltage-gated calcium channels containing the $\alpha_2\delta$-1 or the $\alpha_2\delta$-2 subunit (Marais et al., 2001) which associate with the α_1 subunit to regulate current amplitude (the $\alpha_2\delta$-3 and $\alpha_2\delta$-4 also exist but are not affected by gabapentin). The high threshold L type and P/Q type channel are thought to be affected by gabapentin, although it does not appear to affect the subtypes $Ca_v1.2$ $Ca_v2.1$ (Lacinova, 2005). The $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits are highly expressed in small diameter DRG neurons (Yusaf et al., 2001), furthermore, expression of the $\alpha_2\delta$-1 subunit is significantly upregulated (presynaptically) in the spinal dorsal horn ipsilateral to spinal nerve ligation and is thought to be involved in the tactile allodynia associated with this injury (Li et al., 2004)

Potassium

Voltage-gated potassium channels (K_v) open following depolarisation to allow outward currents and are responsible for the hyperpolarisation phase of an action potential and stabilising the cell membrane potential and can therefore, in principle, suppress activity of nociceptive neurons. There are many types of K_v channels, which are divided into 12 families (K_v1-12), with each family having more than one member (Ocana et al., 2004). The K_v4 channel has received recent attention as a target for pain plasticity. Decreases in the outward (A-type) currents regulated by this channel have been shown to increase neuronal excitability (Hu and Gereau, 2003). Mice deficient in the K_v4.2 channel show enhanced basal pain responses to a number of sensory tests due to increased excitability of dorsal horn neurons and also an altered second phase formalin response. Following intraplantar formalin administration in these knockouts, a gradual increase in nocifensive behaviour in the second phase is believed to be due to increased firing, which in wild-type animals is attributable to ERK (extracellular signal-regulated kinase) activation and subsequent phosphorylation of the K_v4.2 channel (see Figure 1.4), leading to its inhibition (Hu et al., 2006). ERK is considered to be involved in sensitisation in the dorsal horn following injury by increasing excitability, and this study provides a potential mechanism for this enhanced excitability. Due to the electrical abnormalities associated with injured neurons, the involvement of voltage-gated potassium channels has been studied in a number of pain states. Following CCI, for example, K_v 1.1, 1.2, 1.4, 2.2, 4.2, and 4.3 mRNA levels are all downregulated ipsilateral to injury which suggests the potential involvement of such changes in the generation of the hyperexcitability associated with chronic pain states (Kim et al., 2002).

1.4.5 Peripheral sensitisation

Lowering the activation threshold of the nociceptor terminal at the periphery will increase its excitability in response to a given stimulus (see Figure 1.3.). This can occur by modulating the properties of the various ion channels and ionotropic receptors present on the membrane of the nociceptor terminal. Tissue injury and

inflammation leads to the production of inflammatory mediators such as bradykinin, NGF, prostaglandins and histamine which can increase the sensitivity of the nociceptor terminal via their interactions with metabotropic receptors, to initiate subsequent signal cascades that lead to the phosphorylation of ion channels (Woolf and Salter, 2000; Julius and Basbaum, 2001; Ji and Woolf, 2001).

Figure 1.3

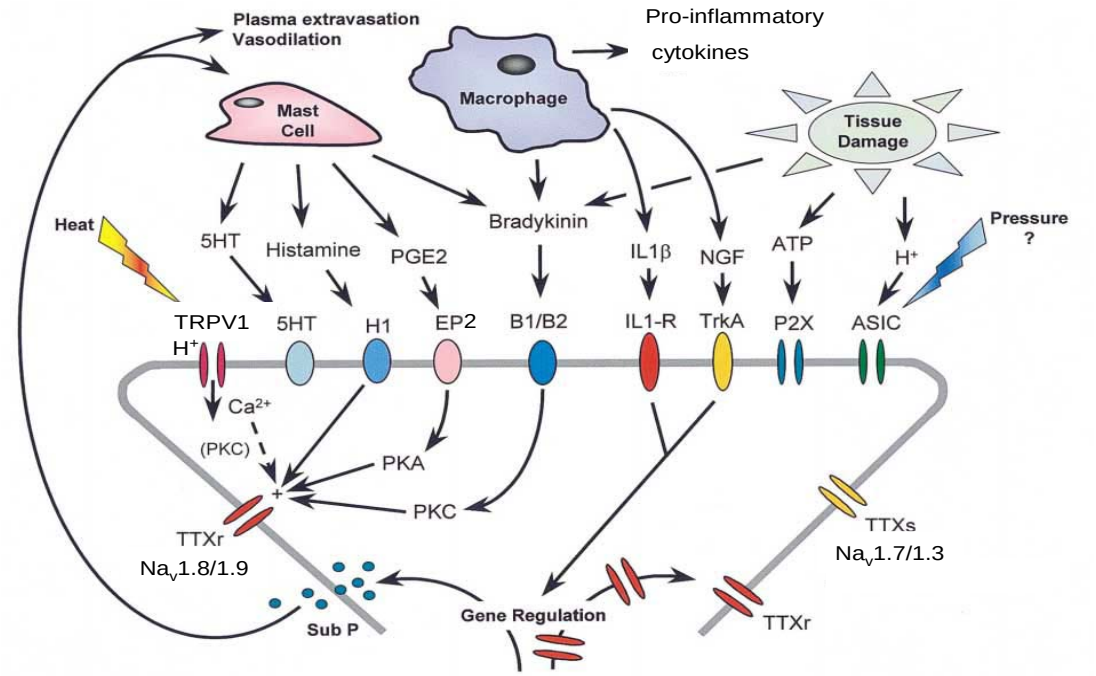


Figure 1.3. Diagrammatic representation of the receptors, and their ligands, found on the peripheral terminals of nociceptive neurons, and their responses to tissue damage/inflammation which includes activation of PKA and PKC which phosphorylate TTX-R sodium channels, lowering their activation threshold, and therefore sensitising the peripheral terminal (Costigan and Woolf, 2000).

1.5 Neurotransmission in nociceptive circuits: Glutamate

Glutamate is the main excitatory neurotransmitter in the dorsal horn and is released from the terminals of peripheral afferents onto second order dorsal horn neurons following stimulation. Glutamate acts on ionotropic glutamate receptors which include NMDA, AMPA and kainate receptors and also on metabotropic glutamate

receptors (mGlu1-8) (Bleakman et al., 2006). AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and kainate receptors mediate fast excitatory transmission in the dorsal horn (the majority is carried out by AMPA receptors), as the NMDA receptor is blocked by Mg^{2+} in a voltage-dependent manner and this functional inhibition can only be removed upon sufficient depolarisation of the postsynaptic membrane (Mayer et al., 1984). As the main excitatory pathway in nociception, much attention has recently been focused on the role of glutamate, its receptors and the resulting second messenger events and signal cascades downstream of receptor activation, in the development and maintenance of persistent pain states. In particular, research has been focussed on the mechanisms responsible for enhancing synaptic efficacy within dorsal horn neurons, a process that is termed central sensitisation (see 1.8.2).

1.5.1 AMPA receptors and their molecular interactions

AMPA receptors are tetramers made up of a combination of the subunits GluR1-4, (Hollmann and Heinemann, 1994) which confer the pharmacological properties of the receptor. The receptor subunits are able to interact with different cytoplasmic proteins via their intracellular C-terminal tails, which vary in length and contain multiple sites for potential regulatory phosphorylation (Lee, 2006). GluR1 and GluR4 subunits have long cytoplasmic tails, GluR3 is short, and GluR2 exists as 2 alternative splice variants which can be either short (GluR2S) or long (GluR2L) (Malinow, 2003). Calcium permeability of the receptor is dependent on the subunit composition, those containing GluR 1/3 or 4 make up calcium permeable receptors, the presence of GluR2 reduces calcium permeability and those receptors lacking GluR2 have been implicated in plasticity, particularly in response to peripheral inflammation (Millan, 2002; Vikman et al., 2008). AMPA receptors are responsible for the majority of fast excitatory transmission and changes to their numbers and functional properties within the synapse may contribute significantly to central sensitisation. Of particular interest are the mechanisms responsible for the trafficking of specific AMPA receptor subunits to the synaptic membrane and the subsequent clustering of these receptors and their associated proteins within the postsynaptic

density (PSD). A number of studies indicate an essential role for synaptic insertion of the GluR1 subunit in particular, in the generation of long-term potentiation (LTP), which is thought to be orchestrated by CaMKII (calcium/calmodulin-dependent protein kinase) and PKA (Roche et al., 1996; Zamanillo et al., 1999; Hayashi et al., 2000). Additional studies suggest that receptors formed of subunits with long cytoplasmic tails (GluR1 or GluR4), together with a GluR2 subunit, are capable of activity dependent trafficking to the synapse to facilitate LTP, and are subsequently replaced with GluR2 or 3 subunits (Shi et al., 2001).

PSD-MAGUKS (membrane-associated guanylate kinases) are known to play an important role in the synaptic clustering of AMPA receptors in the mature spinal cord. PSD-95 and/or PSD-93 (MAGUKS that are known to interact directly with NMDA receptor subunits) have been shown to be crucial in regulating AMPA mediated synaptic transmission (Elias et al., 2006). Elias et al (Elias et al., 2006) demonstrate the compensatory capacity of the post synaptic density proteins by showing that AMPA mediated synaptic transmission is only impaired when *both* PSD-93 and PSD-95 are knocked out, which helps to explain why previous studies focusing only on PSD-95 mutations, fail to show any effect on AMPA mediated synaptic transmission (Migaud et al., 1998). Such an absence of PSD-93 and 95 may result in compensatory changes in remaining members of the PSD-MAGUK family, such as SAP 97 and SAP 102. SAP102 is upregulated in double PSD-93 and 95 knockouts in the adult and is vital for transmission in immature synapses where PSD-93 and 95 levels are low and increase developmentally (Elias et al., 2006). SAP 97 binds directly to the GluR1 subunit, (Leonard et al., 1998) (unlike the other PSD-MAGUKS which bind via transmembrane AMPA receptor regulatory proteins, (TARPS) such as stargazin) and has been shown to rescue the reduced AMPA mediated synaptic transmission caused by knock*down* of PSD-95 (Schluter et al., 2006) which implies its importance in the targeting of the GluR1 subunit to active zones.

1.5.2 Kainate Receptors

High threshold primary afferent (Aδ and C-fibre) input is required for postsynaptic activation of the kainate receptor in the dorsal horn (Li et al., 1999), which suggests that kainate receptors may only involve pro-nociceptive stimuli and this receptor is becoming much more widely studied to determine its role in chronic pain states (Chizh, 2002). Kainate receptors are tetrameric structures formed from a combination of high affinity glutamate binding KA1 and KA2 subunits and the lower affinity GluR5-7 subunits. The composition of these subunits will confer the functional properties of the resulting channel. Furthermore, the GluR5 and GluR6 subunits undergo post transcriptional mRNA editing of a single residue (from glutamine to arginine) (Sommer et al., 1991), which reduces the Ca^{2+} permeability of the channel and therefore the excitability of the cell and subsequent calcium-dependent cellular events such as receptor trafficking and phosphorylation. Conversely, an increase in the unedited forms of these subunits would increase channel conductance and lead to neuronal hyperexcitability, and recently an increase in the unedited GluR6 subunit has been implicated in inflammatory hyperalgesia (Guo et al., 2002).

1.5.3 NMDA receptors and their molecular interactions

The NMDA receptor (NMDAR) is an ionotropic glutamate receptor permeable to Ca^{2+} and Na^{+} ions. The NMDAR is made up from a combination of two NR1 subunits and two regulatory NR2 (A-D), or NR3 (A or B) subunits, which confer the functional characteristics of the receptor and can be modified by phosphorylation (Monyer et al., 1992). NMDA receptor activation requires both the presence of glutamate in the synapse, together with the co-agonist glycine or D-serine, as well as strong depolarisation of the postsynaptic membrane in order to remove the Mg^{2+} block and allow influx of Ca^{2+} ions (Wroblewski et al., 1989; Bleakman et al., 2006; Ascher and Nowak, 2009). Non-NMDA glutamatergic activation, via AMPA or kainate receptors, can sufficiently depolarise the membrane to relieve the Mg^{2+} block; additionally, changes in receptor characteristics can reduce its affinity for the

magnesium ion. Once the NMDA receptor is activated, Ca^{2+} ions are allowed to enter and can, directly or indirectly, activate many downstream effectors such as the kinases; PKA, PKC, CaMKII and tyrosine kinases such as Src, which are able to phosphorylate the receptor and subsequently alter its properties (Lee, 2006). The relationships between different NMDA subunits and associated proteins have been studied in detail to elucidate the specific pathways that lead to receptor phosphorylation, trafficking to the synapse and changes in receptor composition (Wenthold et al., 2003; Prybylowski et al., 2005; Groc et al., 2009). The NR1 subunit has phosphorylation sites that can be targeted by PKA or PKC (Tingley et al., 1997). Both phosphorylation at these sites and increased synaptic insertion have been associated with the hyperalgesia and allodynia generated in response to peripheral inflammation (Caudle et al., 2005; Yang et al., 2009).

CaMKII is one of the major kinases involved in LTP and synaptic plasticity (Barria and Malinow, 2005; Lee et al., 2009) and binds to the C-terminal tail of the NR2B subunit with high affinity and also to the NR2A subunit, but with a lower affinity (Strack and Colbran, 1998; Barria and Malinow, 2005). A recent study has shown that a switch from NR2B to NR2A expression, consequently reducing CaMKII binding at the synapse, is able to reduce LTP (Barria and Malinow, 2005). Furthermore, inhibition of CaMKII activity leads to a reduction of NR2B levels at the synapse and a reduction in LTP (Gardoni et al., 2009). The important interactions between the NR2B subunit and CaMKII in facilitating LTP are heavily dependent on the postsynaptic density, particularly PSD-95 which allows the clustering of these proteins at the synapse and indeed, a disruption of NR2B/PSD-95 binding can also disrupt LTP (Gardoni et al., 2009). Due to their importance in modulating synaptic efficacy, these crucial postsynaptic interactions have been studied in chronic pain states. Garry et al 2003 (Garry et al., 2003) have shown a reduction in pain behaviour following nerve injury in mice expressing a truncated PSD-95. These authors have also shown a decreased association of CaMKII with NR2A/B subunits in these mice which is thought to be responsible for the lack of sensitivity. The tyrosine kinase, Src is perhaps one of the most recent NMDA associated kinases to be targeted in the chronic pain field. A recent study has shown that by disrupting the association between Src and NMDA complexes using a blocking peptide, both inflammatory and

neuropathic pain behaviour is reduced (Liu et al., 2008). These authors showed that tyrosine phosphorylation of the NR2B subunit occurs in response to formalin injection and that the NMDA-dependent second phase behavioural response can be prevented by blocking Src and therefore NR2B subunit phosphorylation. Similarly, hypersensitivity as a result of peripheral inflammation (CFA) or nerve injury was also suppressed by the blocking peptide (Liu et al., 2008).

The mitogen-activated protein kinase (MAPK) family include extracellular-regulated kinases (ERK), p38, and c-Jun N-terminal kinase (JNK) which all form part of different signalling cascades. Activation of the NMDA receptor is required to activate the ERK pathway (Ji et al., 1999). Activated ERK, phospho-ERK (pERK), has been implicated in the enhancement of nociceptive pathways as its levels are markedly increased in response to noxious stimuli, furthermore inhibition of the ERK kinase, MEK, is able to reduce the second phase of the formalin response (Ji et al., 1999). The exact mechanism for ERK involvement in central sensitisation is unknown, although recent studies suggest that phosphorylation of the Kv4.2 channel by ERK could be a possible mechanism (see 1.4.4) (Hu et al., 2006).

1.5.4 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors, of which there are 8 subtypes (mGluR 1-8), which are divided into 3 groups depending on their subsequent signalling pathways and their structural and pharmacological characteristics (Neugebauer, 2002). To briefly summarise, Group I (mGluR 1 and 5) receptor activation leads to the activation of phospholipase C (PLC), and subsequent release of calcium from intracellular stores, whereas group II (mGluR 2 and 3) and group III (mGluR 4/6-8) are negatively coupled to adenylate cyclase, so inhibit cAMP (cyclic adenosine monophosphate) formation and PKA activation (Neugebauer, 2002). Generally, activation of group I mGluRs increases neuronal excitability and can enhance ionotropic glutamate receptor activity (Bleakman et al., 1992) by mechanisms such as receptor phosphorylation, and groups II and III are generally inhibitory. However, in terms of nociceptive processing, the overall effect on dorsal horn output will depend on the location of the receptor, as group II or III

mGluRs could have excitatory effects if located presynaptically on inhibitory interneurons, for example.

1.6 Neurotransmission in nociceptive circuits: GABA and Glycine

Gamma-aminobutyric acid (GABA) and glycine are the main inhibitory neurotransmitters in the central nervous system. GABA acts on either ionotropic $GABA_A$ receptors or metabotropic $GABA_B$ receptors, both of which can be found in the dorsal horn on primary afferent terminals, as well as on inhibitory interneurons (Millan, 1999). Activation of $GABA_A$ receptors allows Cl^- to run down its concentration gradient, which is normally inwards, resulting in hyperpolarisation of the cell. Similarly, $GABA_B$ activation initiates a signal cascade which ultimately leads to a decrease in Ca^{2+} currents and an increase in K^+ currents to hyperpolarise the cell and reduce transmitter release (Millan, 1999). Glycine receptors are mainly located postsynaptically to primary afferents in the dorsal horn and are permeable to Cl^- ions and their activation similarly, leads to hyperpolarisation of the neuron (Millan, 2002). Chloride homeostasis is regulated by the K^+ Cl^- co transporter (KCC2). A reduction in expression levels of KCC2 would disrupt the transmembrane anion gradient, shifting GABA-mediated signalling from hyperpolarising to depolarising. Studies have shown that downregulation of KCC2 in dorsal horn neurons occurs ipsilateral to peripheral nerve injury and is implicated as a potential mechanism underlying hyperexcitability of lamina I neurons (Coull et al., 2003). Further studies from Coull *et al* (Coull et al., 2005) have shown that ATP-stimulated microglia are involved in the shift in the anion gradient in GABAergic neurons within lamina I following nerve injury. The study shows that stimulated microglia transplanted into naïve rats are able to induce a shift in GABA responses from hyperpolarising to depolarising which is accompanied by a reduction in paw withdrawal threshold. BDNF release from microglia is thought to be mechanism responsible for this interaction between activated microglia and GABAergic neurons, (Coull et al., 2005) and blockade of this signalling may provide a novel analgesic mechanism.

In chronic pain states there is evidence to suggest that GABAergic interneurons can co-express other neurotransmitters such as the endogenous opioid, enkephalin, acetylcholine, (see 1.7), or glycine (Millan, 1999). These inhibitory interneurons can be activated by both nociceptive (Aδ and C-fibres) and non-nociceptive (Aβ) fibres and can subsequently affect dorsal horn output via the connections they make with projection neurons (Willis and Coggeshall, 1991). These mechanisms form the basis of what was described by the Gate Control Theory, proposed by Ronald Melzack and Patrick Wall in 1965 (Melzack and Wall, 1965). According to the Gate Control Theory, activation of Aβ fibres by light touch activates inhibitory interneurons in the dorsal horn which in turn inhibit output from projection neurons, potentially reducing the signal from nociceptive neurons and therefore pain perception. The fine balance of these inhibitory mechanisms can be shifted or disrupted in cases of chronic pain states such as nerve injury or inflammation (Zeilhofer, 2008). Two of the main mechanisms put forward by which inflammation or nerve injury have been shown to lead to disinhibition within the dorsal horn are by prostaglandin-mediated inhibition of glycinergic signalling or apoptotic cell death of GABAergic neurons, respectively (Ahmadi et al., 2002; Moore et al., 2002). Following peripheral inflammation, the enzymes necessary for the production of prostaglandins, and specifically PGE_2, are upregulated in the spinal cord (Claveau et al., 2003). PGE_2 acts postsynaptically at the EP2 receptor to ultimately activate PKA which leads to phosphorylation-induced inhibition of a specific subunit of glycine receptors (GlyRα3) that are expressed in the superficial dorsal horn (Ahmadi et al., 2002). Peripheral nerve injury has been shown to activate caspase, a protease essential for apoptotic cell death, in dorsal horn neurons, which have been shown to be largely GABAergic interneurons (Scholz et al., 2005). The authors demonstrated that the mechanism for the induction of cell death is mediated by glutamate and activation of NMDARs, and suggest that the excitotoxic levels of glutamate released post-injury may be responsible for the cell death of GABAergic interneurons (Scholz et al., 2005).

Ultimately, the result of such disinhibition would be persistent input from primary afferents onto WDR neurons lacking inhibitory tone, which may result in central sensitisation (see 1.3.3 and 1.8.2).

1.7 Modulation of dorsal horn transmission

The intensity and duration of the output from projection neurons in the dorsal horn is dependent on the balance between excitatory primary afferent input, local excitatory and inhibitory circuits within the dorsal horn and supraspinal descending input. These systems can all be modified following nerve injury or inflammation, to tip the balance towards hyperexcitability of dorsal horn neurons and subsequent central sensitisation and the generation of pathological pain states.

1.7.1 Primary afferent input and local spinal circuits

The peptide neurotransmitters SP and CGRP are co-released from C-fibres along with glutamate and can further enhance glutamate release (Kangrga and Randic, 1990) thereby potentially contributing to the depolarisation of the postsynaptic neuron. SP receptors (NK_1 receptors) are located on lamina I neurons that are nocispecific projection neurons and belong to the spinothalamic and spinoparabrachial tracts (see 1.3.4). It has also been proposed that these neurons are essential in the development of central sensitisation in response to injury, as ablation of the receptor is able to reduce hyperalgesia (Mantyh et al., 1997; Khasabov et al., 2002). Transmission of nociceptive signalling by projection neurons can be modulated within the dorsal horn by endogenous opioids, such as endorphins, enkephalins and dynorphins (Millan, 2002). Opioid receptors include the mu (μ), delta (δ) and kappa (κ) receptor subtypes, and are present within the superficial dorsal horn. These receptors are G protein-coupled receptors which are negatively coupled to adenylate cyclase to increase K^+ currents and decrease Ca^{2+} in order to decrease excitability (Millan, 2002). The mu and delta opioid receptors have previously been shown to be colocalised with SP and CGRP in the peptidergic population of C-fibres. However, recent studies (Scherrer et al., 2009) indicate a differential expression of mu and delta opioid receptors in primary afferents with the mu subtype primarily localised within peptidergic C-fibres and the delta receptor localised within the non-peptidergic sub-class of C-fibres, as well as within myelinated Aδ fibres. Furthermore, the mu and delta opioid receptors are implicated

in the modulation of heat and mechanical stimuli, respectively (Scherrer et al., 2009). In addition to the 3 main subtypes of opioid receptors, the ORL-1 (Opioid Receptor-Like 1) is a G protein-coupled receptor, negatively coupled to adenylate cyclase with a sequence closely related to those of the opioid receptors (Meunier, 1997). Activation of the ORL-1 receptor by its endogenous ligand, nociceptin (Meunier et al., 1995; Reinscheid et al., 1995), inhibits N-type voltage-gated calcium channels, and activates potassium channels, which results in a reduction in neurotransmitter release from primary afferents (Henderson and McKnight, 1997). The expression of both nociceptin and the ORL-1 receptor has been shown to occur within many regions associated with nociceptive processing, such as the thalamus, PAG, and also the spinal cord and in small to medium-sized DRG neurons, where these proteins have been shown to be upregulated following nerve injury or inflammation (Chen and Sommer, 2006). The precise role of this receptor and its ligand in nociceptive behaviour is still subject to debate. The name "nociceptin" was originally given as it was thought that this ligand induced hypersensitivity based on results from intracerebroventricular administration of the ligand, and also from knockdown studies (Chen and Sommer, 2006). More recent studies have in fact shown an opposite, anti-allodynic, effect when nociceptin is given intrathecally to neuropathic rats, an effect which can be potentiated when given with morphine, suggesting an opioid-dependent mechanism of action at the spinal cord (Courteix et al., 2004). Subsequent studies, however have indicated that the nociceptin/ORL-1 system may have an anti-opioid influence, as ORL-1 antagonists administered systemically, are able to potentiate morphine anti-allodynia in chronic pain, suggesting that the upregulation of the nociceptin/ORL-1 system observed in chronic pain states may contribute to the poor efficacy of opioids in treating chronic pain (Khroyan et al., 2009). The discrepancies and confusion that have arisen regarding the nociceptin/ORL-1 system may therefore be partly down to the presence of underlying pain states, different routes of administration or agonists and antagonists, and mechanisms of action at spinal or supraspinal sites, but its role remains as yet unclear.

1.7.2 Descending control of dorsal horn neurons

The noradrenergic and serotonergic pathways from the RVM (see Figure 1.1) are the main descending pathways to the dorsal horn and their overall effects on output can be either inhibitory or facilitatory. Inhibitory mechanisms reduce dorsal horn output by hyperpolarising superficial dorsal horn neurons, inhibiting transmitter release from primary afferents and also increasing inhibitory tone via GABAergic and glycine interneurons (Millan, 2002). Noradrenaline acts both pre- and post synaptically via the α_2 adrenoceptor, which is negatively coupled to adenylate cyclase, to reduce transmitter release from primary afferents, and also to prevent firing of projection neurons (Li and Zhuo, 2001; Millan, 2002; Pan et al., 2002; D'Mello and Dickenson, 2008) both of which will serve to reduce dorsal horn output. The α_2 adrenoceptor agonist clonidine is able to alleviate the allodynia induced by nerve injury and has been used to treat neuropathic pain clinically (Eisenach et al., 1995). The mechanism responsible for the analgesic effect of clonidine is not fully understood. Studies have indicated that intrathecal clonidine may exert its analgesic effect by α_2R-mediated depolarisation of cholinergic neurons to increase spinal acetylcholine, which has been shown to reduce glutamatergic transmission in the dorsal horn by inhibition of glutamate release from primary afferents and excitatory interneurons, and potentiation of GABAergic tone via activation of different muscarinic receptor subtypes (Pan et al., 1999; Pan et al., 2002; Zhang et al., 2007; Gassner et al., 2009). Conversely, α_1 adrenoceptors are positively linked via G protein-coupled receptors to voltage-gated calcium channels and so their actions on neuronal activity are largely excitatory, which given their location on projection neurons and primary afferent terminals, along with increased expression following peripheral nerve injury, indicates a pro-nociceptive role (Millan, 2002).

Serotonergic pathways are a further source of descending modulation on dorsal horn neurons, also originating from the RVM in the brainstem (see Figure 1.1). The majority of these neurons terminate in superficial laminae of the spinal cord (Millan, 2002). Depending on the receptor targeted, the effect on output can either be facilitatory or inhibitory. Of the 14 subtypes of serotonin receptor the most abundant in the spinal cord are 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, and 5-$HT_{3,}$ as identified by binding

studies (Millan, 2002). 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} are expressed in the superficial dorsal horn and are negatively coupled to adenylate cyclase and lead to the opening of K^+ channels to exert an inhibitory effect on transmission (Millan, 2002). Studies also suggest a role for spinal 5-HT_7 receptors in mediating morphine induced anti-nociception, as this opiate-induced analgesia can be blocked by intrathecal administration of antagonists for the 5-HT_7 receptor (Dogrul et al., 2009). Furthermore, systemic administration of 5-HT_7 receptor agonists, is able to dose-dependently reverse capsaicin-induced mechanical hyperalgesia (Brenchat et al., 2009), although the basis for the spinal mechanism proposed to explain this is not fully understood.

The 5-HT_3 receptor is an ionotropic cation channel located predominantly on C-fibres in lamina I and activation of this channel by descending serotonergic pathways can facilitate nociceptive transmission, particularly in inflammatory pain states (Millan, 2002; Yoshimura and Furue, 2006). The pro-nociceptive pathway responsible for this facilitation of nociceptive signals is thought to originate in the dorsal reticular nucleus (DRt) of the medulla (Brenchat et al., 2009). Nociceptive inputs to spinal dorsal horn neurons and the subsequent rostral propagation of these signals by NK_1 receptor-positive projection neurons is able to activate descending serotonergic pathways which act on 5-HT_3 receptors to enhance excitability in the spinal cord (Suzuki et al., 2002; Rahman et al., 2007). Disruption of this loop, either by ablation of NK_1-positive projection neurons, brainstem lesions, or antagonism of spinal 5-HT_3 receptors is able to reduced the hyperalgesia and allodynia associated with chronic pain states (Rahman et al., 2007) and could therefore be seen as a potential target for therapeutic intervention.

As discussed above, information about painful stimuli is not simply transmitted directly to the brain from the spinal cord unchanged; importantly, nociceptive signals can be modulated depending on circumstances. In cases of persistent activation of dorsal horn neurons, in chronic pain states for example, modulation of output occurs as a result of a number of plastic changes within the spinal cord which can often outlast the original injury and give way to pathophysiological responses.

1.8 Plasticity in nociceptive circuits in the spinal dorsal horn

1.8.1 Signalling and second messenger pathways

Many of the modulatory effects necessary for plasticity in nociceptive neurons and their pathways arise as a result of complex signalling cascades and second messenger systems. Upon activation of membrane receptors such as the NMDAR, a number of intracellular responses are initiated in response to the influx of calcium. These include activation of protein kinases which are responsible for the phosphorylation of receptor subunits and therefore altered function, thus much attention has been focused on these pathways and their involvement in synaptic plasticity, and specifically the maintenance of persistent pain states (Ji and Woolf, 2001; Lee, 2006). However, the variety and complexity of these signal transduction mechanisms makes selecting specific molecules for possible therapeutic targets difficult.

1.8.2 Synaptic plasticity: LTP and central sensitisation

Nociception is subject to modification based on previous injuries and experiences, such modifications may be long-lasting and can be pathological in cases of chronic pain states. It is believed that the modification of synaptic efficacy following peripheral injuries primarily takes place within the dorsal horn of the spinal cord and is therefore termed central sensitisation. LTP and LTD (long term depression) are physiologically opposing mechanisms of synaptic plasticity. As previously discussed (1.5.3) there are a number of protein kinases implicated in NMDA-dependent potentiation of postsynaptic signalling. Conversely, various protein phosphatases (PP1, PP2A and PP2B), are implicated in LTD, and it has been proposed that protein phosphatases and protein kinases have higher and lower affinities for Ca^{2+}, respectively, and so small increases in calcium would favour protein phosphatase activity and LTD, whereas a large increase in Ca^{2+} would promote protein kinase activation and the induction of LTP (Lee, 2006). It has been suggested that central sensitisation shares some of the molecular mechanisms associated with LTP in the hippocampus (Ji et al., 2003), which has long been implicated in learning and

memory (Morris et al., 1986). LTP is a long lasting increase in synaptic strength which results from high frequency repetitive stimulation and in order for this to be induced and maintained, changes must occur within the synaptic structure and function; such changes may include altered presynaptic transmitter release and/or postsynaptic responsiveness to these transmitters (Ji and Woolf, 2001). Central sensitisation is defined by the IASP as "an enhanced responsiveness of nociceptive neurons in the CNS to their normal afferent input" (Merskey and Bogduk, 1994). Importantly, this facilitatory input can come from more than one source whereas LTP results from homosynaptic input, furthermore, nociceptive input at dorsal horn neurons is usually low frequency and therefore potentiation of these neurons is more likely to arise from low frequency heterosynaptic input (Ji et al., 2003). It is important to specify when discussing pain mechanisms that central sensitisation refers to the altered response of nociceptive neurons receiving afferent input, as there is a need to take into account the influences of inhibitory interneurons as well as descending input (see 1.3.2 and 1.3.3) within the dorsal horn. Both LTP and central sensitisation have been shown to require NMDA receptor activation and subsequent increases in intracellular calcium (Lynch et al., 1983; Morris et al., 1986; Woolf and Thompson, 1991), although voltage-gated calcium channels, AMPA receptor activation and release from intracellular Ca^{2+} stores can also contribute to a rise in intracellular Ca^{2+} concentration. Such increases in intracellular Ca^{2+} concentration can lead directly or indirectly to the activation of protein kinases such as CaMKII, PKC and PKA, which can mediate changes in synaptic potentiation by phosphorylation of glutamate receptors at specific sites to alter their physiological properties and also to promote trafficking of receptors to the postsynaptic membrane (Ji and Woolf, 2001). Specifically, phosphorylation of the GluR1 subunit of the AMPA receptor and the NR2B subunit of the NMDA receptor have received much attention recently for their roles in enhancing synaptic efficacy (Wang and Salter, 1994; Boehm et al., 2006; Kopec et al., 2007). In addition to increasing synaptic efficacy by modulation of receptors, increased insertion of AMPA receptors will also enhance synaptic transmission and such mechanisms are being extensively studied with a view to understand and potentially develop ways to treat the hypersensitivity associated with chronic pain.

Figure 1.4

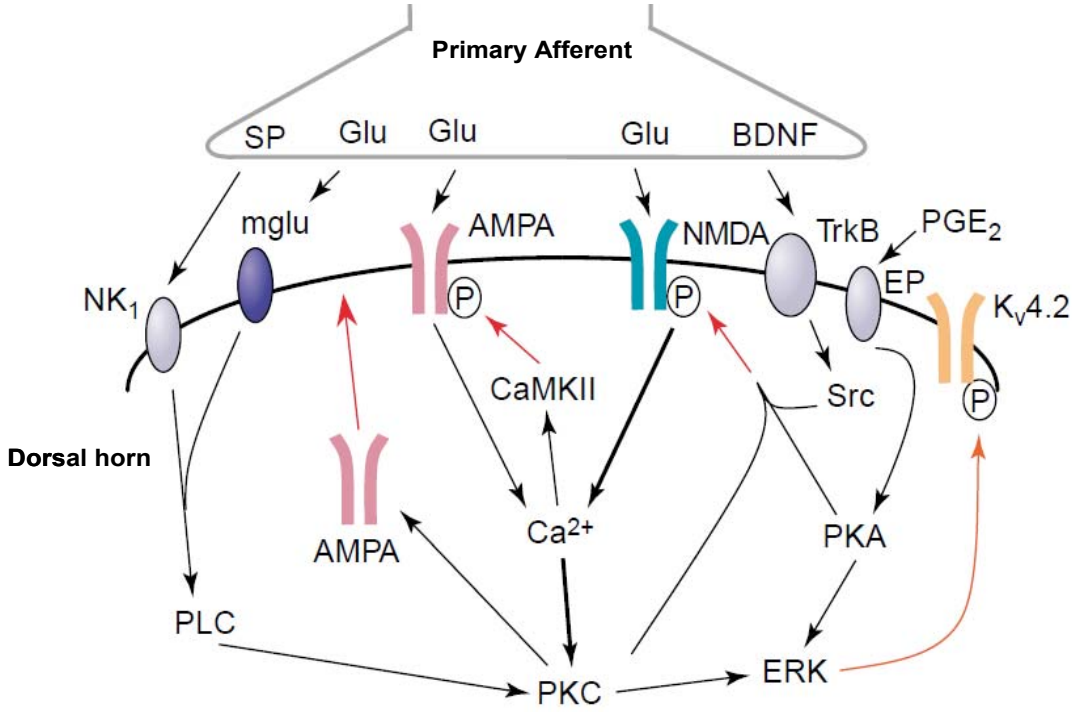


Figure 1.4. Central sensitisation in the dorsal horn. Glutamate (Glu), Substance P (SP) and brain-derived neurotropic factor (BDNF) are released from primary afferents. Glutamate acts upon NMDA, AMPA or metabotropic glutamate receptor (mglu) and SP and BDNF activate the G-protein-coupled neurokinin 1 (NK_1) receptor or the tyrosine kinase receptor TrkB, respectively. Calcium and downstream signalling activates kinases, CaMKII, Src, PKA and PKC which phosphorylate (red arrows) AMPA and NMDA receptors to increase synaptic efficacy. ERK also phosphorylates (orange arrow) the $K_v4.2$ K^+ channel which results in inhibition of the outward current regulated by this channel to regulate the hyperpolarisation phase of the action potential. (Ji et al., 2003)

1.9 Pain in the preterm and newborn

Early-life pain such as that likely to be experienced by human pre-term neonates in neonatal intensive care units is poorly understood and requires further investigation. It was previously thought that due to an under-developed nervous system, neonates lack conscious awareness of medical procedures carried out and are therefore given little or no analgesia (Anand et al., 2005). Recent studies are now not only indicating changes in response to neonatal inflammatory (Ruda et al., 2000; Walker et al., 2003;

Sternberg et al., 2005) and neuropathic pain (Lee and Chung, 1996; Howard et al., 2005), but also providing evidence for a conscious awareness, indicating higher-level processing and potential suffering (Bartocci et al., 2006; Slater et al., 2006).

1.9.1 Development of nociceptive inputs

At birth, the development of the somatosensory system is ongoing. This is true of many mammalian species, including in humans (especially in preterm neonates) and also the laboratory rodent. Postnatal development of the sensory system allows early life events to influence somatosensory sensitivity due to the ongoing organisation of sensory connections and receptor expression in the CNS and peripheral nervous system (Fitzgerald, 1995; Alvares and Fitzgerald, 1999; Fitzgerald, 2005). One of the major differences between the adult and neonatal nociceptive system in the rat is that A-fibres have a strong input onto lamina II neurons in the neonate, which normally only receive C-fibre input in the adult, the possible exception being in sensitised states. A-fibres are the first to reach the dorsal horn at around E13 and are able to evoke action potentials in the dorsal horn from P3 (Jennings and Fitzgerald, 1998). These fibres begin to withdraw from lamina II over the first 3 postnatal weeks (Fitzgerald et al., 1994) and so any insult to the nervous system during this time may affect this redistribution within an already "sensitive" system. Peptidergic C-fibres reach Lamina II at E18-19 (Mirnics and Koerber, 1995) and the non-peptidergic subclass of C-fibres arrive later at around P5 (Benn et al., 2001). However, although peptidergic C-fibres can be observed in the dorsal horn early in development, evidence suggests that they do not mature in terms of their expression of SP and CGRP at primary afferent terminals until P21 and the SP receptor, NK_1, does not become localised to the superficial dorsal horn until the second postnatal week (Charlton and Helke, 1986; Marti et al., 1987). Additionally, the sodium channel $Na_v1.8$ is not expressed in C-fibres at adult levels until P7 (Benn et al., 2001). Together these developmental changes to C-fibre phenotype indicate that adult-like responses to nociceptive stimuli may not be possible until at least the second or third postnatal week.

As discussed previously (1.5), fast excitatory transmission in the dorsal horn is mediated by AMPA and kainate receptors. AMPA receptors are highly expressed in the dorsal horn at birth, particularly GluR 1, 2 and 4 subunits (Jakowec et al., 1995). Furthermore, compared to adult levels, the ratio of GluR2 subunits to GluR1, 3 and 4 subunits is lower indicating the likelihood a higher influx of Ca^{2+} in neonatal dorsal horn neurons, due to greater numbers of calcium-permeable AMPA receptors. The kainate receptor subunits GluR 5 and 6 are also expressed in the superficial dorsal horn at birth, with expression peaking at P10, and declining by the 3rd postnatal week (Stegenga and Kalb, 2001). Furthermore there is a developmental switch in the mRNA editing of peripheral GluR5 subunits of the kainate receptor (see 1.5.2), in the non-peptidergic population of C-fibres (Lee et al., 2001). By postnatal day 7, most of the GluR5 subunits are in the edited form (Lee et al., 2001) which have a low permeability to Ca^{2+}. Together with the enhanced excitation in the dorsal horn early in development, the developmental switch in GluR5 subunit editing in C-fibres is thought to allow for growth and the formation of nociceptive connections with lamina II dorsal horn neurons, as this switch occurs at the same time as C-fibres begin to terminate within the superficial dorsal horn (Lee et al., 2001). As with other excitatory glutamate receptors, NMDAR subunit expression in the spinal cord is developmentally regulated. NR1 expression declines from P2 to P10 and is relatively low in adult tissue in comparison to neonatal levels, NR2B follows a similar expression profile to NR1 in that its expression is decreased developmentally, with high levels detected in the substantia gelatinosa at P2 and P10 and a decline in expression by P22 and in adult tissue. NR2A is expressed at low levels at P2 but expression increases by P22 (Stegenga and Kalb, 2001).

Generally, all three subtypes of ionotropic glutamate receptor, AMPA, kainate and NMDA, are highly expressed in the neonatal dorsal horn compared with that of the adult. It has been suggested that glutamatergic transmission in the neonate, and Ca^{2+} influx through the GluR1-containing AMPA receptor, may play a role in activity-dependent growth and maturation of neurons (Inglis et al., 2002). Furthermore, the ongoing reorganisation of glutamate receptors postnatally, and the subsequent receptor characteristics brought about by these changes, may make these synapses more prone, compared to mature synapses, to long-lasting modification following

disruption to their usual expression profiles, this could be brought about by injury and the consequential changes in signalling within nociceptive circuitry.

In terms of modulation and output from the dorsal horn, projection neurons are present in the superficial dorsal horn prenatally, although representation of paw stimulation in the rodent is not present in the somatosensory cortex until P12, and interneurons mature slightly later at around P20, (Thairu, 1971; Bicknell, Jr. and Beal, 1984), while descending inhibitory connections such as the serotonergic pathways, are not developed until the second postnatal week (Galineau et al., 2004). Endogenous inhibitory control by interneurons is limited in the early postnatal period, and is dependent on GABAergic transmission, and particularly the $GABA_A$ receptor, with glycinergic signalling appearing by P8 (Baccei and Fitzgerald, 2004).

The functional significance of these immature inhibitory connections coupled with strong A-fibre input and high Ca^{2+} influx through ionotropic glutamate receptors, together with little or no descending inhibitory control, makes the immature dorsal horn much more excitable than that of the adult (for a summary of changes see Figure 1.5).

As discussed above, the first 3 postnatal weeks are a crucial time for reorganisation and maturation of dorsal horn input, with large diameter A-fibres occupying the same laminae as polymodal nociceptive C-fibres. The functional consequence of overlap of non-noxious and noxious input postnatally, means that discrimination between the two types of stimuli is difficult, receptive field sizes are large and coupled with a lack of endogenous inhibition during this period, neonatal nociception is potentially greater than that of an adult. Interruption of this normal restructuring of inputs with postnatal injury may have the potential to induce long-term changes to nociceptive processing.

Figure 1.5

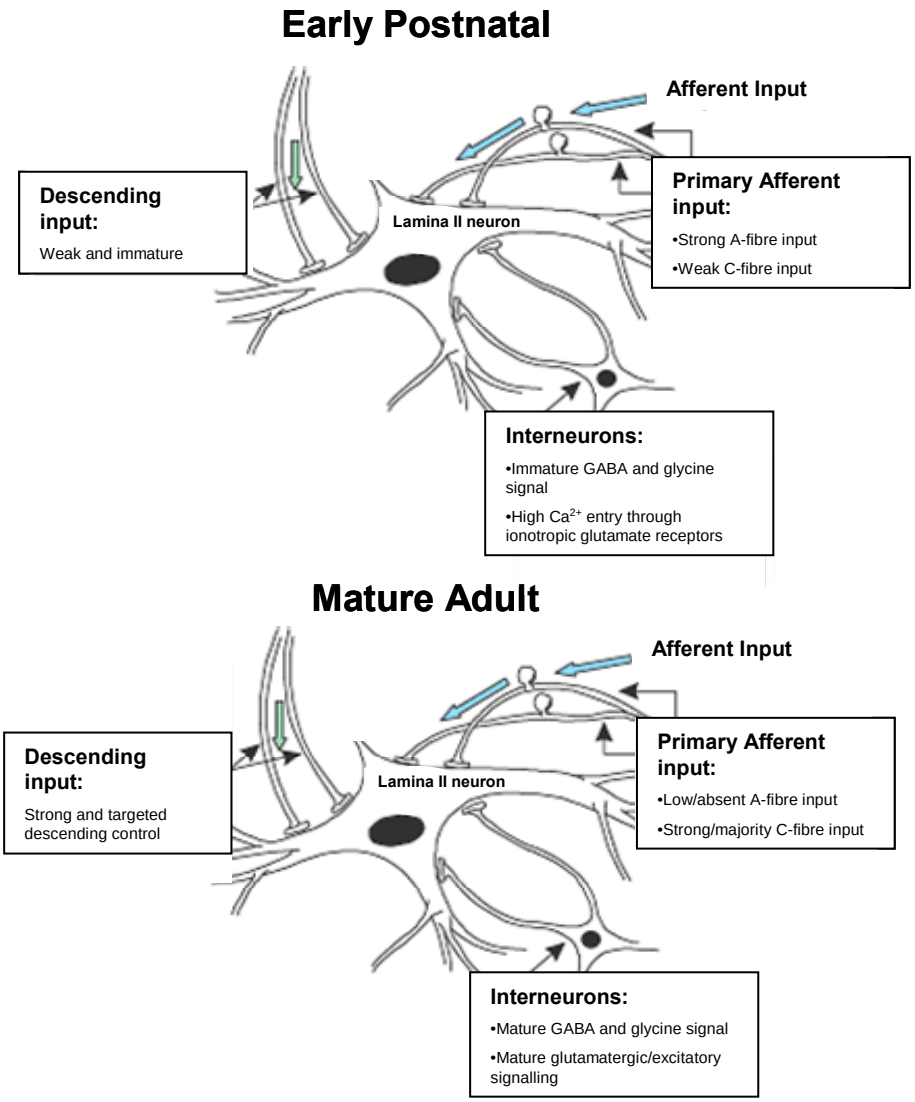


Figure 1.5. Diagrammatic representation of developmental changes which take place in superficial dorsal horn neurons from early postnatal life (top) to adulthood (bottom). Blue arrows represent primary afferent input, green arrow represent descending pathways originating from the brainstem. Information summarised from,(Fitzgerald, 2005).

1.10 Early life events and programming

The process by which external events during development exert long-term changes in physiological processes is termed "programming". As well as the obvious need to identify the mechanisms which may be involved in human programming of pain sensitivities as a result of early life injuries (discussed 1.9), programming in domestic species is becoming increasingly more apparent (Jarvis et al., 2006) and poses a range of as-yet unanswered questions over animal welfare. This is especially important in the pig farming industry where not only are pregnant sows socially stressed from mixing with unfamiliar sows, but offspring experience early-life pain, with tail-docking and tail biting occurring soon after birth (Compassion in World Farming (CIWF), 2009). The effects of this combination of aversive early life stress and postnatal pain need to be further explored both in pigs and appropriate laboratory models to understand changes that may occur in physiology and behaviour. A full appreciation of the overall impact of early life adverse events will require not only assessment of the first generation but also in subsequent generations to examine the effects of the maternal behaviour of the affected daughters and heritability.

1.11 Animal welfare

As previously mentioned (1.1), pain in animals can often be difficult to assess due to an inability to communicate verbally. To overcome this, most of the current assessments of pain in animals are based on efferent responses and can be defined as follows:

"Animal pain is an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; (note there may not be any damage) it changes the animal's physiology and behaviour to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery." (Molony, 1997)

Due to the distress and potential incapacitating effects evoked by pain, it has become a major animal welfare concern. Of particular importance are chronic pain states which may develop following tissue or nerve damage or inflammation, which can

occur in association with disease states, but also may occur following the absence of adequate pre-emptive analgesia prior to surgery or routine husbandry procedures such as castration and tail docking (Compassion in World Farming (CIWF), 2009). The long-term consequences of such procedures, on both sensory processing and affective behaviour, have not yet been fully investigated. Furthermore, the possible synergistic effect of adverse prenatal experiences and postnatal pain on animal welfare needs further study.

1.12 Prenatal stress

1.12.1 The Hypothalamic-Pituitary-Adrenal axis

Stress can be defined as a potential or existing threat to an organism's homeostasis, and the physiological stress response is an attempt to counteract such threats. There are many pathways within the brain that are activated in response to stress and which converge onto corticotropin-releasing-hormone (CRH) neurons within the PVN of the hypothalamus to activate the hypothalamo-pituitary-adrenal (HPA) axis, (Figure 1.6) (Johnstone et al., 2000; Russell et al., 2001; Kudielka and Kirschbaum, 2005). The HPA axis begins with CRH release from the PVN. CRH, along with arginine vasopressin (AVP), are released from PVN terminals at the median eminence into the hypophysial portal blood system where they are transported to the anterior pituitary to activate CRH type I and AVP (V1a) receptors, respectively, to synergistically stimulate release of adrenocorticotropic hormone (ACTH) from corticotrophs within the anterior pituitary. ACTH is released into the systemic blood, and acts upon the adrenal cortex to stimulate glucocorticoid (i.e., cortisol in humans and corticosterone in mice and rats) release. Glucocorticoids exert many metabolic and physiological effects throughout the body and also modulate negative feedback at the level of the hippocampus, pituitary and PVN to reduce further ACTH and therefore glucocorticoid release. The most widely studied effects of glucocorticoids are mediated genomically upon binding to intracellular glucocorticoid receptors (GRs), which once activated, translocate to the nucleus to bind to the glucocorticoid response element (GRE) to mediate gene expression, thus having the capability to

produce the diverse effects mentioned previously and described below with reference to prenatal stress. Glucocorticoids are also able to exert their effects via non-genomic mechanisms which are independent of GRs and may be mediated through direct interactions of the steroid or through interactions with additional partner proteins. These non-genomic mechanisms are able to have rapid effects as they are independent of transcription and protein synthesis (Haller et al., 2008). As mentioned, GRs along with the higher-affinity mineralocorticoid receptor (MR), mediate negative feedback of the HPA axis. The highest concentration of MRs in the brain is found in the hippocampus where they are bound by low levels of glucocorticoids and are important for tonic regulation of basal HPA axis activity. GRs are found throughout the brain, but at high concentrations in the hippocampus, PVN and pituitary where they mediate feedback control of stress-induced glucocorticoid secretion (de Kloet and Reul, 1987), although it is thought that MRs are also important for stress-induced negative feedback control (Dallman et al., 1989).

It is important to consider the developmental profile of the components of the HPA axis and its associated structures when investigating the potential adverse outcomes of perinatal stressors. The embryonic development of fetal rat and human brain areas is reviewed by Weinstock, 2001 (Weinstock, 2001). An essential component of the HPA axis, the PVN, does not develop until the later stages of embryonic life in the rat (E13-15) and is responsive to maternal corticosterone by E15 (Fujioka et al., 1999; Fujioka et al., 2003), whilst the hippocampus and therefore feedback control and regulation of the HPA axis, is not fully established until after birth (CA E16-20, dentate gyrus P3-15). In the rat brain glucocorticoid receptors are present in the last prenatal week, with GRs appearing first and MRs appearing in the hippocampus slightly later, with levels remaining low until after birth when they begin to increase (Diaz et al., 1998). Given the developmental profile of the components of the stress response in the rat, it is clear that the last prenatal week is a particularly sensitive window for this system and studies have shown that maternal stress during this time has adverse effects on offspring HPA axis activity and also anxiety behaviour (Weinstock, 2008). However, it must be noted that the developmental profile of the

HPA axis is highly species-specific and therefore different windows may exist where perinatal programming of this system is most likely to occur.

Figure 1.6

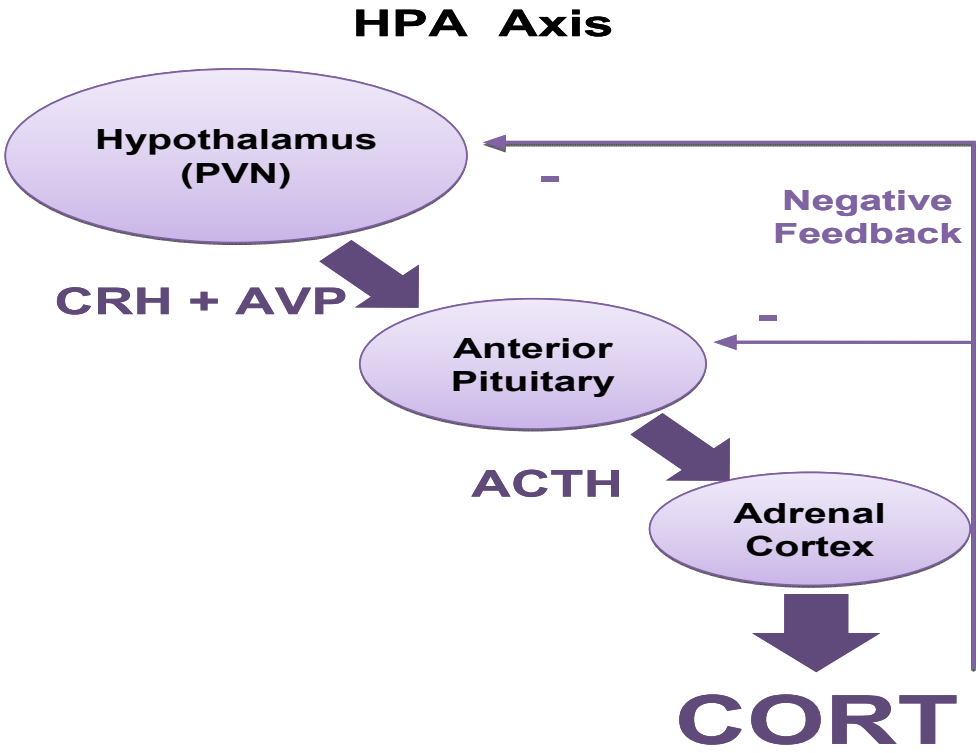


Figure 1.6. Diagrammatic representation of the HPA axis. Corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from the paraventricular nucleus (PVN) of the hypothalamus and act upon CRH type I and V1a receptors in the anterior pituitary to stimulate release of adrenocorticotropic hormone (ACTH). ACTH is released into the systemic blood and stimulates corticosterone/cortisol (cort) secretion from the adrenal cortex which is then able to feedback at the level of the pituitary, hypothalamus and hippocampus (see 1.11.1 for detail).

1.12.2 Perinatal protective mechanisms

The uterine environment is designed to help protect the developing fetus and ensure offspring are well prepared to face their external environment at birth. It has been well documented that exposure to excess stress hormones (glucocorticoids) during prenatal life can have adverse effects on stress responsiveness in adulthood and may

also predispose to a number of other diseases in later life (Clark, 1998; Welberg and Seckl, 2001; Seckl, 2004; Moritz et al., 2005). It therefore makes sense, from an evolutionary perspective, that the maternal HPA axis undergoes a period of hyporesponsivness in late pregnancy (Brunton and Russell, 2008). The mechanisms responsible for this hyporesponsive period have recently been identified (Brunton et al., 2009) and are due to endogenous opioid inhibition of stress-induced noradrenergic input to the PVN, which presynaptically reduces noradrenaline release and subsequent CRH secretion. This opioid-mediated inhibition of the stress response is thought to be induced by allopregnanolone, a progesterone metabolite which is found in the brain at high levels during pregnancy. These mechanisms protect the fetus from circulating glucocorticoids during critical developmental windows, during which fetal programming is most likely to occur (Johnstone et al., 2000; Russell et al., 2001). Further protection is provided in the form of placental 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) which converts maternal corticosterone to inactive 11- dehydrocorticosterone. However, in cases of persistent or highly stressful situations, these protective mechanisms may fail, allowing increased levels of maternal glucocorticoids to reach the fetus and have the aforementioned effects (Welberg et al., 2005). In addition to *in utero* protection, rodents have a postnatal stress hyporesponsive period occurring between postnatal days 4 and 14, which also seems to be under maternal control by mother-infant interaction (Levine et al., 1991; Levine, 1994). Given the relative immaturity of a rat pup at birth, this postnatal protection from high glucocorticoid levels is essential to prevent adverse effects on neuroendocrine and CNS development.

1.12.3 Prenatal stress – models and effects

Much of the data obtained from the effect of prenatal stress in humans comes from retrospective studies where the stressor has been in the form of natural disasters, war or terrorist attacks and also in the form of more personally directed stressors such as tensions or abuse at home or in the workplace. The impact of such stressors on offspring is, not surprisingly, varied and includes elevated basal and stress induced HPA axis activity, alterations in development and functioning of organs, as well as

behavioural abnormalities in childhood and adulthood (Weinstock, 2001; Welberg and Seckl, 2001; Kapoor et al., 2006; Yehuda and Bierer, 2008; Mastorci et al., 2009). In the laboratory rat, it is possible to investigate the effects of fetal glucocorticoid exposure by administering synthetic glucocorticoids such as dexamethasone or increasing endogenous glucocorticoids by exposing the pregnant female to a highly stressful situation. Exposure to dexamethasone, prenatally, leads to low birth weight, elevated stress responsiveness and diseases such as hypertension and hyperglycemia in the adult (Benediktsson et al., 1993; Clark, 1998; Seckl, 2004; Moritz et al., 2005). Other models of prenatal stress aim to increase endogenous corticosteroids by activating the maternal HPA axis. One of the most commonly used prenatal stressors is restraint, which has many documented detrimental effects on offspring including changes in pain behaviour (Butkevich et al., 2006; Darnaudery and Maccari, 2008). The mechanism by which the maternal stress response can affect its offspring is still unknown; there is evidence to support both pre and postnatal maternal influences. Barbazanges *et al.* (Barbazanges et al., 1996) carried out one of the first studies to implicate elevated maternal corticosterone as the potential mechanism for producing prenatally programmed offspring. The study shows that restraint stress in the last week of pregnancy, which is known to increase corticosterone levels, leads to a prolonged stress-induced corticosterone response in adult offspring, as well as a decrease in MR, (which are important for negative feedback control of glucocorticoid secretion, see section 1.12.1). Both of these outcomes can be prevented by removing maternal corticosterone using adrenalectomy and can be reinstated in adrenalectomized mothers when supplementing with doses of corticosterone to mimic stress-induced levels. Other studies have investigated the effects of maternal stress on 11β-HSD2, as another possible mechanism to account for the effect the maternal stress response can have on offspring. It has been shown that restraint stress is capable of reducing both placental and fetal 11β-HSD2 (Mairesse et al., 2007), thus reducing the level of protection against elevated maternal glucocorticoids and also increasing the availability of corticosterone in pups themselves. In addition to these direct effects of maternal corticosterone on the development of offspring's HPA axis *in utero*, gestational stress may also lead to a reduction in maternal behaviour (Baker et al.,

2008), which can negatively affect postnatal programming of offspring's stress responsiveness. Increases in maternal care in the form of licking and grooming have been shown to increase hippocampal GR expression, and therefore negative feedback sensitivity, via alterations to the epigenome (changes in gene expression without altering underlying DNA sequence) through functional modulation of the GR gene promoter region (Weaver et al., 2004). This may help to explain how postnatal maternal interactions are able to induce long- lasting changes in offspring HPA axis responsiveness. It has been known for a number of years that postnatal handling is able to reduce HPA responsiveness and stress induced anxiety (Levine, 1962; Meerlo et al., 1999) and it is thought that the reason for this is due to the increased maternal attention the pup receives once it is returned to the home cage following handling. Furthermore, cross-fostering studies, which result in increased attention towards the fostered pup, have been shown to reverse the negative HPA axis effects of prenatal stress (Maccari et al., 1995). In light of the data discussed, it is important to consider the range of complicated interactions which take place between a mother and her offspring in the perinatal period when studying programming of HPA axis responsiveness.

1.13 Hypothesis

We hypothesise that the combination of a nerve injury and inflammation will result in enhanced sensitivity when compared to the component single injuries. Furthermore, we hypothesise that these injuries carried out in early life will lead to long-lasting changes in nociceptive processing, which may be further exacerbated by prenatal stress.

1.14 Aims

This thesis will explore the long-term consequences of chronic pain using a novel pain model in laboratory rats that is designed to reflect combined neuropathic and inflammatory injury. Such a combination would occur together clinically as a result of post-surgical infection for example, and also following husbandry practices that

could potentially compromise animal welfare, such as tail-docking in the piglet. We will characterise this novel model in the adult, before taking it to the neonate to explore its effects on nociceptive responsiveness compared to those elicited by component inflammatory or neuropathic injuries. Further we will evaluate whether any of these early life injuries are able to bring about long-term plasticity in nociceptive processing pathways that may be manifest as amplification or altered characteristics of the response to noxious challenge in later life. Finally, we will ask whether prenatal stress can programme stress and pain sensitivity of the offspring, either alone or in combination with early life injury such that enhanced endocrine and reflex responses are seen in response to noxious events in subsequent adult life.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, and had received ethical approval from the University of Edinburgh ethical committee. Sprague Dawley rats were used in all experiments and were given access to food and water *ad libitum* and housed in accordance with Home Office guidelines. Ambient temperature and humidity were 21°C and 50% respectively; lighting was on a 12h on: 12h off schedule, with lights on from 07.00h to 19.00h. Rats were normally housed in same sex groups of up to 6 per cage. Pups were weaned between postnatal day (P) 22 and P28 and housed with same sex littermates where appropriate.

2.2 Procedures

2.2.1 Adults

Adult male Sprague Dawley rats (150-200g) were either given an injection of Complete Freund's Adjuvant (CFA), surgery to produce a Chronic Constriction Injury (CCI) or a combination of both injuries to the right hindpaw/hind leg (see detail below, 2.3.1). Rats were tested from 24 hours onwards following CFA and from 6 days onwards following CCI or combined surgery to allow for healing following separation of the muscle bundles.

2.2.2 Neonates

Female Sprague Dawley rats were mated and the male removed from the cage once females were visibly pregnant. At either post partum day (P) 8 (day of birth =P0) or P18 individual litters were divided into experimental and control groups. Control groups were either non-handled pups (naïve controls) which were not tested and remained with the dam at all times, or rats which were anaesthetised (anaesthetic controls) and separated from the dam for the same amount of time as experimental

groups at the time of surgery and also tested at the same time as the experimental groups. Experimental groups were either CCI, CFA or a combination of the two injuries on P8 or P18. Experimental groups and anaesthetic controls were weighed on every day of testing to ensure that pups were all gaining weight and therefore being allowed to feed and were not in a distressed state which may be accompanied by weight loss. Weighing the animals also gave an indication of the developmental stage, as pups from larger litters may be smaller than a pup from a smaller litter, which may affect sensory thresholds.

2.2.3 Prenatal social stress model – Resident/intruder paradigm

Upon arrival into the unit, male and female Sprague Dawley rats were put on a breeding diet, (2019 Teklad, Harlan, UK) which was maintained for the duration of the experiment. Animals were not used until at least a week after arrival into the unit to allow them to settle in.

Residents

Nulliparous 14 week old female Sprague-Dawley rats (n=30) were paired with experienced adult Sprague Dawley males overnight. Females were checked every morning for vaginal plugs, unplugged females were immediately removed from the breeding cage and returned to their original cage until the end of the day so as to limit the window of conception to overnight. The day of detection of a vaginal plug was considered to be Day 0 of pregnancy (E0) and the females were removed and individually housed in clear plastic cages in a separate room. The day of birth was considered as lactation day 1 (P0). Pups were regularly monitored and date of birth and litter size was noted. Females considered to be inadequate mothers or behaving abnormally towards their pups were immediately culled.

Intruders

A second set of nulliparous 14 week old females (n=20) were mated (as above) and the plug date noted. These females were housed in normal breeding cages in a separate room from the residents (see below). Pregnant females with unknown plug dates were excluded from the experiment but allowed to give birth to generate pups for use as control groups; unused litters (i.e., those never exposed to intruders) from the resident group were also added to this control group.

Resident/Intruder social stress paradigm

This paradigm is a well established model of social stress which utilizes maternal defence of pups to exert emotional stress on the female intruder (Neumann et al., 2001). From E16 to E20 (inclusive), pregnant (intruder) females were placed in the cage with a lactating resident (lactation day 2-8) and her pups for a 10 minute period once daily. Experiments were carried out between 09.00h and 12.00h in a separate room from where animals were housed and all lactating (resident) females were allowed to habituate to the testing room for at least 30 minutes prior to the interaction. The interaction was monitored and recorded using Anymaze software (Stoelting C0 Illinois - US) to compare levels of aggression towards the intruder and the interaction was scored as high, medium or low. The intruder was returned to its own cage at the end of each encounter. All residents were between days 2 to 8 of lactation because this is when they are considered to be at their most aggressive and were preferably used at the beginning of this period as maternal aggression seems to tail off by day 8 (Neumann et al., 2001). Intruders were exposed to a different resident on each of the 5 days and each resident was used a maximum of twice per day. Any interactions in which residents did not display maternal aggression and where the intruder did not seem to be submissive were not considered to be stressful to the intruder. A minimum of 4 out of the 5 days (E16-E20) of social stress during the last trimester was required for subsequent offspring to be considered to have been exposed to sufficient prenatal stress for inclusion in our experiments, although basal and stress-induced corticosterone levels are not determined until later in the study.

2.3 Surgery

Rats were anaesthetised by inhalation of an isoflurane/O_2 mixture (Zeneca, Cheshire, UK), 4-5% for induction and 1.5-3% for maintenance. All animals were carefully monitored during surgery/injections; this was especially important in the neonate as maintenance of anaesthesia often required adjustment. Following surgery, adults were monitored until recovery from anaesthesia and for a short time afterwards. Following surgery (and in anaesthetic only controls) at P8 and P18, pups were allowed to recover inside an incubator in a plastic container filled with bedding from the home cage in an attempt to mask smells from the anaesthetic and surgery. Upon recovery from anaesthesia, pups were returned to the home cage and observed until acceptance into the litter by the dam. Animals were all marked using permanent marker pens (whilst anaesthetised) for identification. P8 pups were marked in the same way as adults and P18 pups, with rings around the tail and with a number on the back which was regularly re-marked. These markings were also used to check whether the mother had accepted the pups back into the litter and was licking and grooming the pups as the markings were expected to fade to the same degree in experimental and control animals.

2.3.1 Chronic constriction injury (CCI)

Chronic constriction injury is standardly used in our laboratory as a model of peripheral nerve injury. This model induces reliable tactile allodynia and thermal hyperalgesia which can be quantified over a number of weeks. The surgery involved in this pain model is also much easier to adapt to carry out in young animals (e.g. P8) than some of the other commonly used models detailed in section 1.2.5.

Surgery to produce a chronic constriction injury of the sciatic nerve in rats is well characterised and has previously been described in detail (Bennett and Xie, 1988). Following hair removal and sterilisation of the area (Hibitane 0.05% Zeneca, UK), a small incision was made below the pelvis and the right biceps femoris and the gluteus superficialis were carefully separated to expose the sciatic nerve. Upon isolation of the nerve two loose ligatures were tied around it proximal to the

trifurcation (Figure 2.1.) using 4-0 chromic catgut (SMI AG, Hunningen, Belgium) (5-0 in the neonate – Medgut, South Africa) with a 1mm separation between ligatures. This is an adaptation of the classic CCI model which usually consists of 4 ligatures, we used 2 in both adult and neonate pain models to standardise the injury, as 4 ligatures are difficult to place around the sciatic nerve of P8 animals. We have compared sensory thresholds of adults with 4 ligatures against those with only two and saw no significant differences between the two groups (see Chapter 3, section 3.2.1). The nerve was then carefully placed back into position and the wound closed. Vetbond (Vetbond, 3M, Loughborough, UK) was used to close the wound in P8 animals. For the combined neuropathic/inflammatory pain model, CFA was injected (see 2.3.2) into the right hindpaw after completion of CCI surgery whilst the animal was still anaesthetised.

Figure 2.1

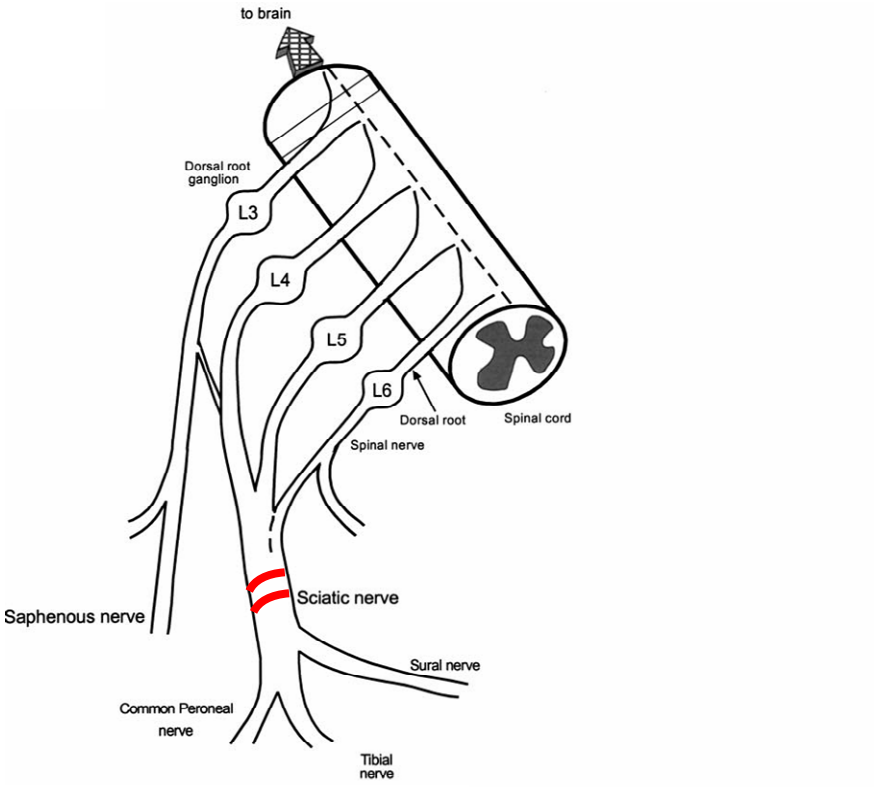


Figure 2.1. Diagram showing sciatic nerve and the terminal branches of the sciatic and saphenous nerves. Red indicates area which is ligated in CCI surgery. (Decosterd and Woolf, 2000)

2.3.2 Complete Freund's adjuvant (CFA)

Following sterilisation (Hibitane 0.05% Zeneca, UK) of the plantar surface of the right hindpaw, 150 µl (adult dose) of 50% CFA (Sigma F5881; 1 mg *Mycobacterium tuberculosis* in 0.85 µl mineral oil and 0.15 µl mannide mono-oleate) in 0.9% saline was injected between the toes and towards the middle of the paw, avoiding the ankle; the needle was withdrawn slowly to minimise leakage. The dose used was similar in adults and neonates as we have found that 1µl/g body weight is sufficient to produce a long lasting inflammatory response in the adult and causes redness and swelling in P18 pups and some swelling when carried out at P8.

2.4 Sensory behaviour tests

Measurements of reflex responses to graded mechanical or thermal stimuli were recorded in conscious animals prior to injury (not possible for P8 injured animals) and regularly post injury to establish a time course of sensitivity. Non-evoked, spontaneous pain behaviour was also measured following injury, in adult animals only. Unfortunately due to the visible changes induced ipsilateral to injury following inflammation and/or nerve injury, it was difficulty to carry out blinded assessments of sensory testing. It is therefore possible that although strict testing methods were in place, there may have been some degree of experimenter bias, which has been previously been shown to result in an overstating of effects (Sackett, 1979; Sena et al., 2007).

2.4.1 General notes and observations

Animals were given 5-10 minutes in their new test environment to allow them to become habituated before testing. In the adult rat, nociceptive sensitivity testing was carried out using both the Hargreaves' test (55°C, Hargreaves' thermal stimulator, Linton Instrumentation, Diss, UK) and von Frey filaments (Stoelting, Illinois, US) (see 2.4.3 and 2.4.2). Due to the continuous growth and development of the neonate, only von Frey testing was carried out as the intensity of the beam of the heat source used in the Hargreaves' test would have needed to be constantly adapted as the animal grew. In particular, the glabrous surface of the hindpaw foot pad becomes larger and the skin thicker as the pup grows, and in younger pups, the beam would cover most of the plantar surface. Therefore, to reduce the numbers of potential variables, only von Frey tests were carried out on the pups. It is only possible to begin testing the neonate on the plantar surface by around P14, before this time pups are much too small for the mesh table used and are barely able to walk as testing depends on a foot withdrawal response and maturity of the motor system, as noted by other groups (Lee and Chung, 1996; Fitzgerald, 2005)

In the case of the P18 groups we could obtain some baseline measurements at P14-P18 (pre-injury); however these measurements may differ over the different days as

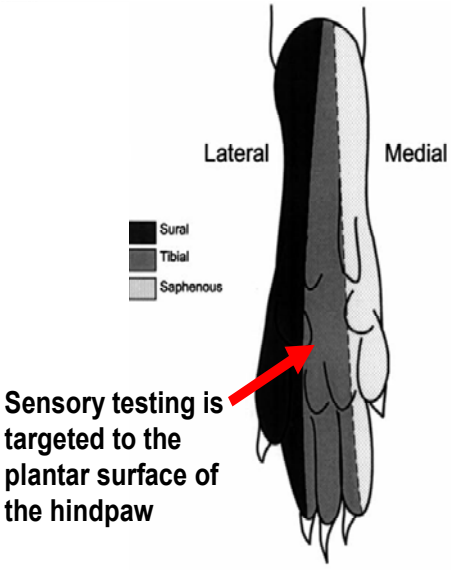
the sensory system continues to develop. We and others (Alvares et al., 2000), have noted a slight increase in baseline threshold on P14. This is the time when the eyes begin to open and the increased threshold may be due to the novel exposure to visual stimuli, during which time the pups become increasingly mobile and perhaps less focused on the sensory test.

2.4.2 Mechanical allodynia – von Frey filament test

Animals were placed on a raised mesh grid and covered with a clear plastic box to contain the animal. von Frey filaments were used to determine the threshold force for withdrawal of the ipsilateral paw compared to the contralateral paw. Filaments were applied to the middle of the plantar surface of each paw from below, (Figure 2.2a) until the filament bent; this was done 6-8 times per filament, in order of increasing force (Figure 2.2b). The filament at which 50% of the applications (i.e. 3 out of 6) resulted in withdrawal was recorded as the paw withdrawal threshold for that particular animal. A minimum of 6 applications were carried out and a maximum force of approx 28g was used to prevent tissue damage. A withdrawal response was considered valid if the animals paw was withdrawn from both the testing platform and also away from the hair itself so as to distinguish from the animal allowing its paw to be simply lifted by the filament; the cut off point of 28g also reduced the possibility of the hair being able to lift the animal's paw. The paw withdrawal threshold (PWT) was measured in grams (g) See table in Figure 2.2 b.

Figure 2.2

a)



b)

Handle	Grams	mN/mm2
3.84	0.692	106.3
4.08	1.202	163.68
4.17	1.479	181.63
4.31	2.041	208.79
4.56	3.63	318.19
4.74	5.495	449.88
4.93	8.511	654.18
5.07	11.749	848.49
5.18	15.136	977.81
5.46	28.84	1609.52

Figure 2.2. Different zones of the plantar surface of the rat hindpaw innervated by the sciatic terminal branches (Swett and Woolf, 1985). Red arrow indicates the area which was tested using graded von Frey filaments (b) and was also targeted using the radiant heat source in the Hargreaves' test. (Decosterd and Woolf, 2000).

2.4.3 Thermal hyperalgesia – Hargreaves' test

Thermal hyperalgesia was measured using the Hargreaves (Hargreaves et al., 1988) plantar test (7370, Ugo Basile, Comerio, Italy). Animals were placed in individual plastic boxes on a glass platform (Figure 2.3.) and allowed to habituate for 10-20 minutes to their new environment. A radiant heat source (Infra red intensity setting 45) was positioned under the platform and focused on the middle of the plantar surface of each hindpaw. The time in seconds at which the animal withdrew its paw was recorded. A cut off time of 20 seconds was imposed to avoid tissue damage. The test was repeated 3 times for each hindpaw with a 5 minute gap between tests to avoid sensitisation. Paw withdrawal latency (PWL) was recorded in seconds and an average was taken for each animal's right and left paw.

Figure 2.3

Figure 2.3. Photograph showing set up for Hargreaves' test to measure thermal hyperalgesia.

2.4.4 Spontaneous pain behaviour

Spontaneous foot lifting (SFL) is thought to be an indicator of ongoing/spontaneous pain (Choi et al., 1994; Djouhri et al., 2006). Following injury, adult animals were placed in individual plastic observation boxes and the cumulative duration of lifting of the ipsilateral hindpaw was recorded over a period of 3 minutes. SFL was often associated with aversive behaviour such as flicking of the ipsilateral paw and is similar to the nocifensive behaviours recorded in the formalin test (see below, 2.4.5). Foot-lifting associated with walking, grooming and other exploratory behaviours was not included.

2.4.5 Formalin test

After behavioural recovery from previous insult (CCI, CFA or the combined injury) adults were subjected to a formalin challenge. Electrophysiological studies (Dickenson and Sullivan, 1987) on dorsal horn neurons have shown that subcutaneous formalin into the receptive field of these neurons results in a biphasic excitatory response, similar to that observed behaviourally. An acute phase at 0-10

minutes post injection is separated by a short interphase of low activity which precedes the longer lasting tonic phase lasting from 20-60 minutes This activity of dorsal horn neurons is thought to be due to peripheral drive from C-fibre activity (Dickenson and Sullivan, 1987).

In animals injured during the neonatal period (at P8), this was carried out at approximately P42, or at recovery from the initial injury. Under isoflurane anaesthesia, 40 µl of a 4% formalin solution, in 0.9% saline, was injected into the plantar surface of the right hindpaw; control groups required for hormone assessments (2.10) received an equal volume of 0.9% saline. Rats were then placed in clear observation boxes to recover from the anaesthesia before recording. Each animal was observed for 60 seconds within a 5 minute window and nociceptive behaviours were recorded during this time using a stopwatch. The cumulative time in seconds was recorded every 5 minutes over 60 minutes or until the termination of nocifensive responses to formalin. Nocifensive behaviours recorded included flinching, flicking, licking or biting of the injected paw. At the end of the test, animals were euthanized by an overdose of carbon dioxide or by conscious decapitation for trunk blood collection.

2.5 Drug administration

Drug effects were assessed in P28 animals by measuring thresholds to mechanical stimulation (see 2.4.2). Pre-drug tests were performed to establish a mean baseline value. Following drug administration, tests were repeated every 10 minutes for 60-80 minutes until measurements returned to those recorded prior to drug application. Trials were carried out blind and the compounds were identified at the end of testing for each batch of animals.

2.5.1 Intrathecal application of drugs

The highly selective NMDAR antagonist 3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid, ((R)-CPP), or saline, were administered into the L5/6 intrathecal space (Figure 2.4) of anaesthetised rats using a 0.5 ml syringe with a 29-gauge

needle (BD Biosciences, Oxford, UK). A tail flick or movement of the hind limbs usually indicated that the needle was correctly positioned prior to injection. Experiments with Pontamine Sky Blue dye (Merck-BDH, UK) were carried out to establish the correct site of injection and ensured that the injected volume of 20µl was sufficient to reach the lumbar enlargement which receives sciatic nerve input. Behaviour testing commenced 15 minutes after injection to allow recovery from anaesthesia and thereby avoid a false analgesic result and also to prevent tissue sensitisation. Previous experiments (Moss et al., 2002) have found complete recovery from anaesthetic by 15 minutes.

Figure 2.4

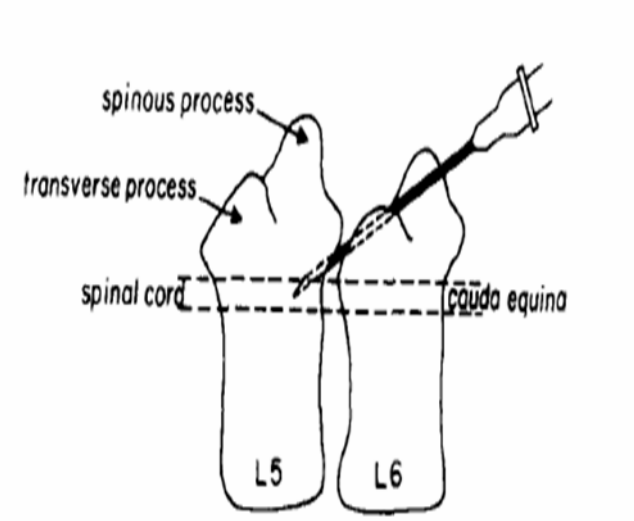


Figure 2.4. Diagram showing insertion of needle into the intervertebral space for intrathecal administration of drugs. (Hylden and Wilcox, 1980)

2.6 Affective behaviour tests

Affective testing was carried out in a room exclusively used for these tests. Each trial was carried out for 5 minutes, as the first 5 minutes of the elevated plus maze (EPM) test is when rats typically exhibit the most avoidance of the open arms (Montgomery, 1955). In our own investigation into an appropriate test length we have compared 10 and 5 minute testing times for both the EPM and open field test and found that the second half of the 10 minute test was typically spent grooming or immobile and did

not contribute any significant findings or differences between groups. The testing arenas (EPM 2.6.1, or open field 2.6.2) were surrounded by a thin opaque white curtain and a thick black curtain to allow control of lighting levels in the test area whilst monitoring behaviour in the same room. Overhead lamps where also used to illuminate the test area where needed. An overhead camera connected to a laptop was used to monitor behaviour from a distance behind a closed curtain and Anymaze software (Stoelting C0 Illinois - US) was used to track behaviour automatically, thus eliminating human error and bias.

2.6.1 Elevated plus maze

The maze was made of metal sprayed black with two opposing open arms and two opposing arms enclosed by walls. The open and closed arms meet in an open centre square and form a plus shape. The plus maze was elevated 45cm from the ground. A smaller version of the maze was constructed for testing pre-weanling rats (P18-22) and was based on dimensions for a mouse elevated plus maze (Stoelting Co Illinois US) (See Figure 2.5a. for diagrams and dimensions). On the day of testing, animals (in their home cages) were brought into the testing room and allowed to acclimatise to the new environment. Dim lighting was used in the test arena and individual animals were removed from their home cages and placed on the centre square of the elevated plus maze facing the open arm and allowed to explore the maze freely for 5 minutes before the animal was returned to its cage. Anymaze software was used to track the animals and therefore monitor various parameters such as time spent in the open and closed arms of the maze automatically. The maze was cleaned thoroughly with ethanol after each trial to remove any olfactory cues (Walf and Frye, 2007).

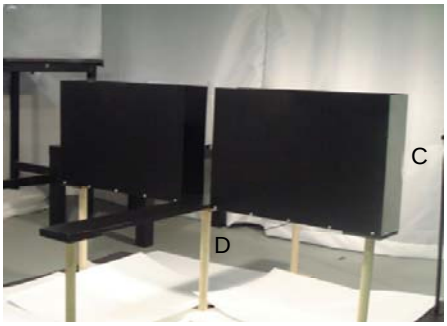
2.6.2 Open field testing

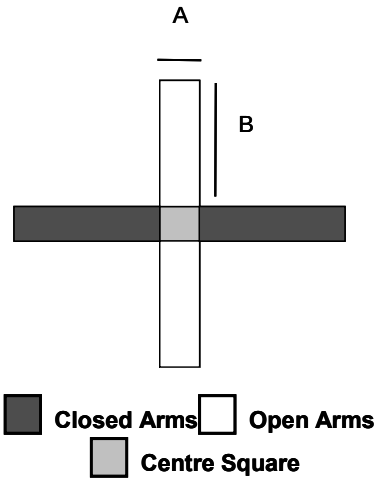
The open field apparatus (based on dimensions from Stoelting Co. Illinois US) consisted of a square arena 40cm by 40cm with 40cm high perspex walls and was lit with overhead lamps. The field was divided into 9 squares, 8 outer squares and 1 central square, using Anymaze software (see Figure 2.5b). On the day of testing,

animals (in their home cages) were brought into the testing room and allowed to acclimatise to the new environment. Individual animals were removed from their home cage and placed in the top left corner of the field and allowed to explore for 5 minutes before being returned to their cage. Anymaze software was used to track the animal's movement and grooming, rearing and defecation were recorded by the tester by holding down a key (G, R or D, respectively) for the duration/number of times the behaviour was observed. Anymaze automatically records parameters of interest such as mobility, distance travelled and entries/time spent in the centre square. After the 5 minute testing period the animal was returned to its home cage and the field was cleaned thoroughly with ethanol after each trial to remove any olfactory cues.

Figure 2.5

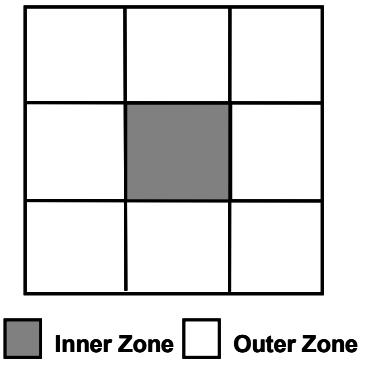
a)





Maze Type	Arm Width (A)	Arm Length(B)	Wall Height(C)	Elevation (D)
Pre-weanling Rat	5cm	35cm	15cm	45cm
Adult Rat	10cm	50cm	40cm	45cm

b)



Open Field Dimensions: 40cmx40cm

Figure 2.5. Photographs and diagrams to show a) elevated plus maze and b) open field test set-up, dimensions and zones.

2.7 Western blotting

Western blotting was used to detect changes in protein levels in DRG or spinal cord tissue in naïve animals and in tissue from different pain models carried out at P8, P18 and in the adult. Tissues were collected at various times post injury, which are specified within the results descriptions.

2.7.1 Tissue preparation

Spinal cord and DRG tissue from L4-L6 were collected from live rats under deep anaesthesia (isoflurane/oxygen, as outlined above, 2.3), or immediately following conscious decapitation. A laminectomy was performed to expose the spinal cord, and tissue removed. Spinal cord from injured animals was hemisected longitudinally down the midline into ipsilateral and contralateral halves, or further divided to isolate only the dorsal horn, this was achieved by opening out the hemisected cord to reveal the dorsal (outer third) and ventral (inner two thirds) grey matter. DRGs were also taken. Naive tissue was processed as whole cord and both the left and right DRGs were collected. To minimise degradation, tissues were collected on ice-cold foil, weighed, and rapidly homogenised in 20 volumes Laemmli lysis buffer (85 % Tris buffer (tris-hydroxymethylaminoethane, 50 mM, pH 7.4, Sigma Chemical Co., UK), 5 % mercaptoethanol (Sigma Chemical Co., UK), and 2 % sodium dodecyl sulphate (SDS, Sigma Chemical Co., UK)). Homogenates were then boiled for 5 minutes to denature proteins and centrifuged for 10 minutes at 11,500g, 10°C, before extracting and aliquoting the supernatant and storing samples at -20°C until needed.

2.7.2 Western blotting procedure

Proteins were separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) using the NuPage XCell *Sure*Lock™ Minicell gel electrophoresis system (Invitrogen, Paisley, UK). Samples (3-5 µl) were mixed with 1 µl loading buffer (0.04%w/v bromophenol blue in glycerol) and loaded into wells on 4 - 12% Bis-Tris NuPage gels (Invitrogen, Paisley, UK). Pre-stained standard

molecular weight proteins (SeeBlue Plus, Invitrogen, Paisley, UK) were run alongside as a guide. Samples were separated by electrophoresis using MOPS running buffer (NuPage, Invitrogen, UK), at 200 V for 50 minutes. Proteins were transferred to a polyvinylidene difluoride Immobilon-P^{SQ} membrane (Millipore, Watford, UK) at 30 V for 90 minutes in transfer buffer (5 % NuPage transfer buffer, Invitrogen, UK, 10% methanol). Transfer and protein loading was assessed by staining membranes with Coomassie blue (0.1 % in 30 % methanol, 10 % acetic acid, GE Healthcare Ltd., UK) and destaining using a solution of 50% methanol, 10% acetic acid in distilled water, followed by scanning. Following removal of the Coomassie blue stain, the membrane was incubated with blocking buffer (5% non-fat dry milk in PBS) overnight at 4^{o}C or 90 min to 2 h at room temperature and probed with primary antibodies (CaMKII: Cat No. 13-7300, 1:250, Zymed. NR2B: Cat No. 32-0700, 1:100-1:250, Zymed. PSD-95: Cat No. 75-028, 1:400, Neuromab) overnight at 4 ^{o}C or for 2 h at room temperature. Following washes with PBS-Tween, blots were probed with appropriate secondary antibodies (anti-mouse: Cat No. AP192P, 1:10,000, Chemicon. Anti-rabbit: Cat No. AP182P, 1:7,500, Chemicon) and detected by peroxidase-linked secondary antibody enhanced chemiluminescence. Blots were also probed for the ubiquitous housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH: Cat No. MAB374, 1:750, Chemicon) for protein level normalization. Image analysis was carried out by scanning the resulting photographic films obtained following chemiluminescence of the western blot. The scans were quantified using Photoshop software to measure greyscale values for a given band. Background measures were also recorded and subtracted from the protein/receptor subunit represented by the band. Greyscale values for receptor subunits were calculated as a percentage of that obtained for GAPDH or the co-immunoprecipitation antibody used.

2.8 Co-Immunoprecipitation

Co-immunoprecipitation was used to investigate changes in protein-protein interactions within the NMDA receptor complex in the spinal cord at peak sensitisation (10 days) following adult pain models.

2.8.1 Tissue preparation

All co-immunoprecipitation experiments were carried out on adult animals (n=7 per pain model group, n=4 for naive groups) at 10 days post injury. Tissue dissection was as described for western blotting (see 2.7.1). Samples were homogenised in 10 volumes of IP (immunoprecipitation) buffer (PBS, pH 7.5 containing 10% glycerol, 1% CHAPS, 0.5% sodium deoxycholate, 1 mM vanadate, 1 mM sodium fluoride, 5-10 mM sodium molybdate) containing 1% protease inhibitor cocktail III (Calbiochem, Merck Biosciences Ltd., Nottingham, UK) and rolled for 1-2 hours at 4 ^{o}C.

2.8.2 Co-immunoprecipitation procedure

Samples were spun at 4°C and 11,500g for 15 minutes and the supernatant extracted and aliquoted. Samples were then pre-cleared for 1 hour at 4^{o}C using protein G sepharose beads (Sigma) at 20µl/ml to remove traces of endogenous antibody, before being incubated overnight at 4 ^{o}C with 40µl/ml of protein G sepharose beads and a combination of NR2A and NR2B antibodies (Cat No. Sc-9056 and sc-9057 respectively, 8.8 µl/ml, Santa Cruz). Controls were incubated with non-immune IgGs from the same species as the co-immunoprecipitation antibody. Following incubation, samples were centrifuged and the supernatant discarded, the remaining beads were washed twice in IP buffer and once in PBS before the addition of equal volumes of Laemmli lysis buffer. Samples were then boiled for 5 minutes, cooled, and stored at -20 ^{o}C. Binding partners within the complex of interest (co-immunoprecipitates of NR2A/B) were detected by western blot, (as described in 2.7.2) running 15-18µl of sample and 2 µl of loading buffer.

2.9 Immunohistochemistry

Immunohistochemistry was carried out to investigate changes and localisation of various proteins in the spinal cord and DRG following pain models in adult and P8 animals.

2.9.1 Tissue preparation

Under deep anaesthesia (isoflurane/O_2), rats were perfused transcardially with phosphate buffered saline (0.1 M phosphate, 0.9% NaCl pH 7.4) containing 100 units heparin/ml, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Tissues were then removed (as for 2.7.1), post-fixed overnight at 4^{o}C in 4% paraformaldehyde, and cryoprotected using increasing concentrations of sucrose in PBS (5% for 1hr, 10% 2hrs, 30% 4hrs). DRGs were then embedded in a freezing/mounting medium (Tissue Tek O.C.T Compound) and stored at -20 ^{o}C (-80 ^{o}C for long-term storage). Spinal cord was stored in PBS-sodium azide at 4 ^{o}C.

2.9.2 Immunohistochemistry procedure

10µm tissue sections were cut on a cryostat at temperatures of -20ºC for DRG and 14ºC for spinal cord and thaw-mounted on poly-L-lysine slides (Merck-BDH, UK). Slides were encircled with a hydrophobic barrier pen (ImmEdge, Vector Laboratories, CA, USA). Slides were blocked for 1 hour at room temperature in block/buffer containing: 10 % normal goat serum (Vector Laboratories, CA, USA), 4 % fish skin gelatin (Sigma Chemical Co., UK), 0.2 % Triton X-100 (Sigma Chemical Co., UK) in 0.1 M PBS. Slides were incubated overnight at room temperature with primary antibodies (ATF-3: Cat No. Sc-188, 1:250, Santa Cruz. NF200: Cat No. N0142, 1:1,500, Sigma) in buffer (same as block). Slides were then washed in PBS and incubated with appropriate secondary antibodies (anti-mouse: Cat No: A11029, 1:750, Molecular probes. anti-rabbit Cat No: A11036, 1:750, Molecular Probes) in buffer (5 % normal goat serum, 2% fish skin gelatin, 0.1 % Triton X-100, 0.1 M PBS). Primary antibody was omitted from some sections to serve as a negative control. ATF-3 is reliably induced following chronic constriction injury and so this particular group served as a positive control. Slides were cover-slipped using Vectashield (Vector Laboratories, USA) and sealed with nail varnish. Sections were visualised under a Leica TCSNT confocal microscope (Leica Microsystems GMBH, Germany). Cells with 50% of the maximum intensity value, as determined by Image J software, were considered ATF-3 positive cells.

2.10 Radiommunossays (RIA)

2.10.1 RIA procedure

Live rats were quickly decapitated and trunk blood was collected into individual Falcon tubes containing chilled 5% EDTA (0.2 ml/100 g body weight) and kept on ice. Samples were then spun at 9,500g for 5 minutes and plasma was separated, aliquoted and stored at -20^{o}C. Plasma ACTH and corticosterone concentrations were determined using commercially available radioimmunoassay kits (Immunodiagnostic Systems Ltd., Tyne and Wear, UK) and following the steps outlined in the manufacturer's protocols. ACTH (adrenocorticotropic hormone) was measured using a two-site immunoradiometric assay (Euro-Diagnostica) which detects only intact ACTH. Corticosterone was measured using a double antibody radioimmunoassay with ^{125}I-corticosterone as the tracer. The sensitivity and the intra-assay variation for the kits of the kits are as follows: ACTH sensitivity 3.0pg/ml and intra-assay variation <4%; Corticosterone sensitivity 7.7ng/ml and intra-assay variation <11%. All samples from the experiment were assayed together. Plasma was diluted to 1:200 for saline treated groups or 1:300 for formalin treated groups (to ensure results were within the range of the test) using the steroid diluent; supplied, concentrations were standardised at the end of the assay.

2.11 Nerve morphology

Morphological changes to the sciatic nerve were investigated following P8 CCI surgery (short duration CCI n=3, long duration CCI n=3) or in naïve animals (n=2). Rats were allowed to recover fully from the initial injury (no difference in ipsilateral paw withdrawal threshold compared to contralateral) and the sciatic nerve was dissected under deep anaesthesia (isoflurane/oxygen) and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer and embedded in araldite. Rats were then culled.

2.11.1 Light microscopy

1μm resin-embedded transverse sections of sciatic nerve (~7-10mm in length) around the site that was ligated were stained with toluidine blue.

2.11.2 G-ratio

To determine changes to myelin thickness in the sciatic nerve, the *G*-ratio of individual axons was measured, using images obtained by light microscopy, by dividing axonal diameter by the total diameter of axon plus myelin sheath.

2.12 Statistical Analysis

For behavioural measures, all values were calculated as group mean +/- SEM at each time point and data were analysed using SigmaStat software (version 2.03) or SPSS (version 14.0). Thermal hyperalgesia was analysed by comparing ipsilateral to contralateral measures using a Student's t-test and post surgery time points were also compared to pre-surgery using a One-Way repeated measures ANOVA (Analysis of Variance) with Dunnett's multiple comparisons test. Mechanical allodynia was analysed using the equivalent non-parametric tests: Mann-Whitney Rank sum test to compare ipsilateral to contralateral and repeated measures ANOVA on ranks (Freidman test) with Dunn's post hoc analysis to compare to pre-surgery. To analyse effects of intrathecal drug application, post-drug ipsilateral measures were compared to pre-drug baseline using the Friedman test with Dunn's post hoc test.
Behaviour in response to formalin and comparing mechanical allodynia in prenatally stressed animals compared to controls was analysed using a two-way ANOVA to identify variation between groups over time. Multiple comparisons were analysed using the Bonferroni post hoc test to compare groups. To compare groups for corticosterone measures from RIAs and also P1 birth weights in prenatally stressed versus control groups Student's t- tests were used. In all cases significance was reached when $p<0.05$.

CHAPTER 3: ADULT MODELS OF CHRONIC PAIN AND THE DEVELOPMENT OF A NOVEL COMBINED PAIN MODEL

3.1 Introduction

As outlined in 1.2.5, an animal model combining both neuropathic and inflammatory pain may yield more realistic and clinically relevant data for a subset of cases. As it was hypothesised that this novel combination of injuries would result in greater sensitivity, the Home Office Animals in Scientific Procedures Division had asked to be notified once 100 animals had been studied and a report was sent detailing preliminary findings. Chronic constriction injury (CCI) is one of the most widely studied models of peripheral neuropathy and results in spontaneous pain, hyperalgesia, and allodynia which are some of the major symptoms suffered by neuropathic pain patients that can be assessed in animals. For these reasons we have chosen to utilise this injury as the neuropathic component of the combined model. The inflammatory component is achieved using Complete Freund's Adjuvant (CFA, see 1.2.5) which provides immune system-mediated long-lasting inflammation that is localised at the site of injection, similar to the type of injury induced upon infection. To characterise this novel combined model we will measure the duration and extent of both evoked and non-evoked pain behaviour, when compared to nerve injury or inflammation alone.

In addition we will utilise the formalin test as a tool to investigate nocifensive responses when the nervous system is challenged following recovery from an initial injury (nerve injury, inflammation or the combined pain model). It should be noted that in this study the administration of formalin into the plantar hindpaw is carried out under anaesthetic to minimise stress, and as a result the first phase behaviour is not seen, however the peripheral input which generates the behaviour observed in the second phase still occurs (Dickenson and Sullivan, 1987) and, importantly, we are able to measure the nocifensive behaviour associated within this phase.

3.2 Results

3.2.1 Mechanical allodynia and thermal hyperalgesia are greater in a combined model of neuropathic and inflammatory pain compared to nerve injury or inflammation alone

In adult rats, the combined pain model, utilising co-administration of CCI and CFA models of neuropathic (nerve injury) and inflammatory pain, respectively, induced sensitisation to mechanical (Figure 3.1 a and b) and thermal stimuli (Figure 3.2), (ipsilateral compared to contralateral) which was longer lasting than that in either of the single models (n=6-9). The difference in paw withdrawal threshold (PWT) in response to von Frey filaments was clearly greater and statistically significant ($p<0.05$) as determined by the Mann-Whitney Rank Sum Test, (ipsilateral compared to contralateral paw), from post-surgical day 7-49 in the combined model, compared to days 10-21 following nerve injury and days 7-42 following inflammation. Additionally, mechanical allodynia was significantly different over time following the combined pain model when compared to the individual pain models. Comparing the difference in PWT (contralateral-ipsilateral) (as plotted in Figure 3.1a) between all three adult pain states revealed a significant effect of treatment (two-way ANOVA, $F=19.86$, $p<0.0001$) with individual group differences identified by Bonferroni post tests occurring between CCI and the combined model at days 7, 14, and 49, and between CFA and the combined model at day 7. There were no time points where significant differences were identified between CCI and CFA. However, similarly, comparing the ipsilateral PWT of the groups over time revealed a significant effect of treatment (two-way ANOVA, $F=18.44$, $p<0.0001$). Contralateral PWT between groups was not significantly affected by treatment. No significant differences in PWT were observed in the combined pain model or the CCI model when two ligatures were used (see chapter 2, section 2.3.1) instead of four ligatures, which are applied in the standard Bennett and Xie CCI model (Bennett and Xie, 1988) (data not shown). For these reasons, to standardise the nerve injury across adults and neonates, due to the technical difficulty associated with using a 4 ligature model in the neonate, all surgery to induce nerve injury used 2 ligatures.

Paw withdrawal latency (PWL) in response to thermal stimuli in the Hargreaves' test (see Chapter 2, section 2.4.3) was significantly decreased ($p<0.05$) as determined by t-test, ipsilateral compared to contralateral paw, from day 6 to 28 in the combined model, days 7-10 following nerve injury and days 1-3 following inflammation (Figure 3.2). Thermal hyperalgesia in the combined pain model also outlasted that of the single injury models when compared to the pre-injured baseline latency. Ipsilateral paw withdrawal latency was significantly different until day 28 post surgery compared to day 10 in nerve injured animals and day 3 in inflamed animals (one-way repeated measures ANOVA with Dunnett's post hoc test to compare to baseline). Two-way ANOVA revealed a significant effect of treatment between ipsilateral PWLs ($p<0.0001$) of all three of the pain models.

Figure 3.1

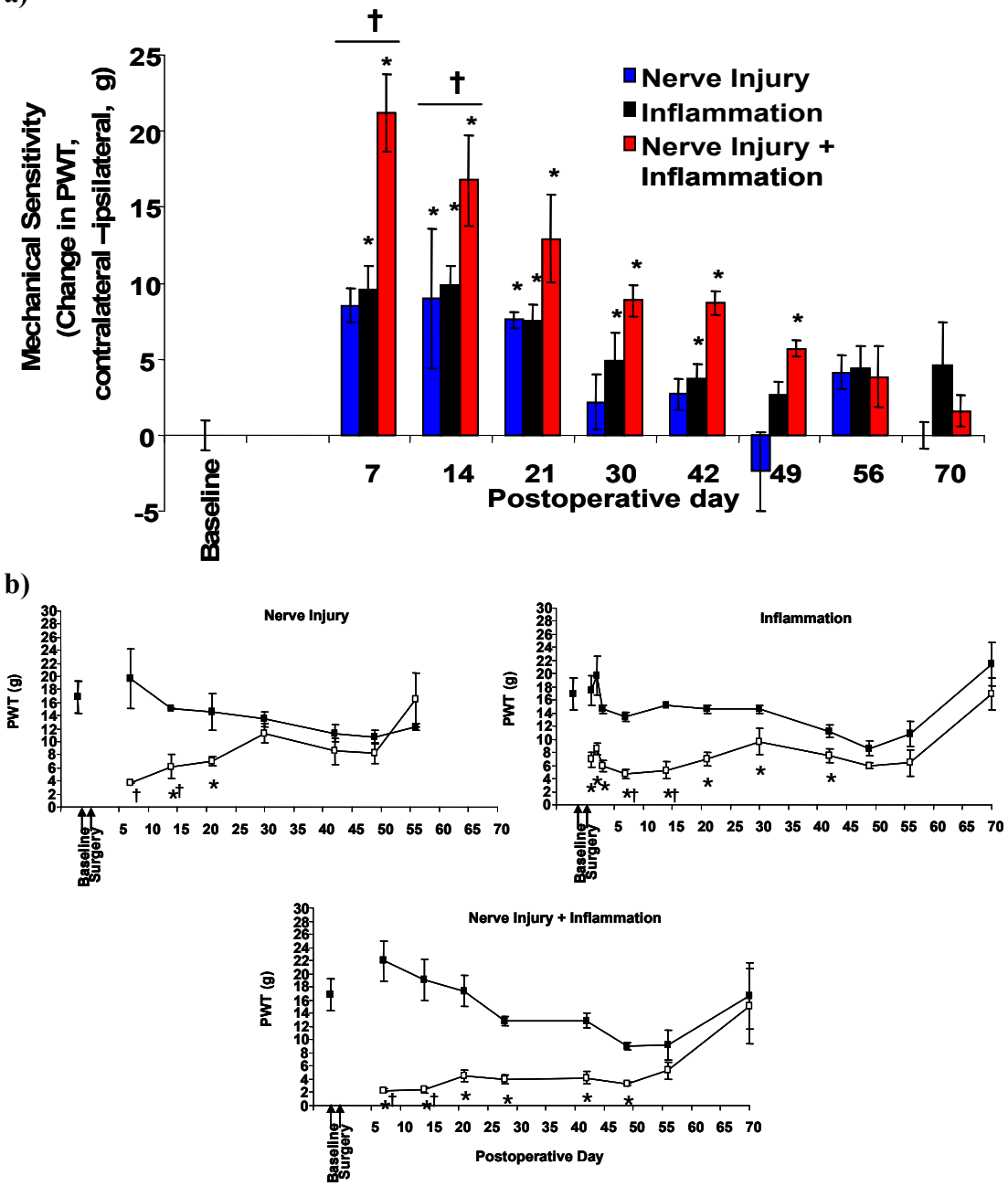


Figure 3.1. Time course of mechanical allodynia in adult rats following injury. Data are expressed as mean±SEMs of a) the difference in paw withdrawal threshold (PWT), following nerve injury (■), inflammation (■) or a combination of these two injuries (■) or b) as absolute values for ipsilateral (open squares) and contralateral (filled squares) paws. Data are obtained from calibrated von Frey filaments (1.2-28.8g). Day of surgery is day 0. (*$p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral paw, from day 7-49 in the combined model, days 10-21 following nerve injury and days 7-42 following inflammation. (†$p<0.05$ Friedman repeated measures ANOVA on Ranks with Dunn's post hoc test to compare post surgery ipsilateral to baseline; n=6 – horizontal bars indicate that all 3 groups differ).

Figure 3.2

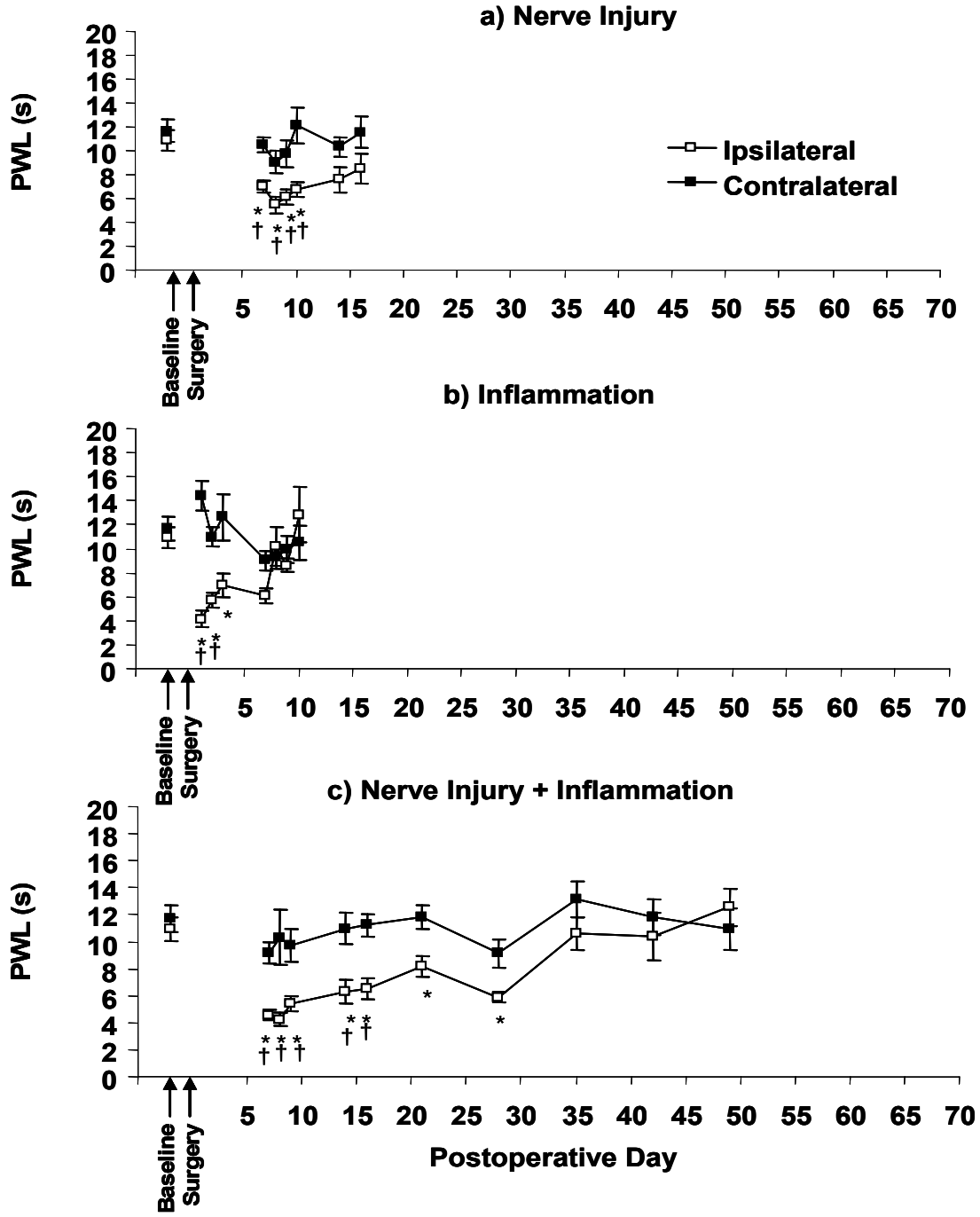


Figure 3.2. Time course of thermal hyperalgesia in adult rats following a) nerve injury, b) inflammation or c) a combination of these two injuries. Day of surgery is day 0. Data are expressed as mean±SEMs for ipsilateral (▫) and contralateral (■) paw withdrawal latency (PWL), measured in seconds. (*$p<0.05$, t-test, ipsilateral compared to contralateral paw, from day 7 to 28 in the combined model, days 7-10 following nerve injury and days 1-3 following inflammation. († $p<0.05$ One-Way repeated measures ANOVA with Dunnett's post hoc test to compare post surgery ipsilateral to baseline; n=6-9).

3.2.2 *Animals with a combination of nerve injury and inflammation display significant levels of non-evoked spontaneous pain behaviour*

During behavioural assessment of the novel, combined pain model it was noted that these animals (n=3-6) displayed significant amounts of spontaneous non-evoked pain behaviour, compared to nerve injury or inflammation alone. For this reason we quantified this behaviour by measuring the cumulative time over which the animal raised its injured paw (spontaneous foot-lifting, SFL) over a 3 minute period (Figure 3.3). We found SFL to be significantly greater compared to nerve injury alone over a number of days (*$p<0.05$, t-test nerve injury compared to nerve injury plus inflammation, days 6-8 and day 12). No SFL of the contralateral paw was observed, and no SFL was observed ipsilateral to inflammation alone (data not shown).

Figure 3.3

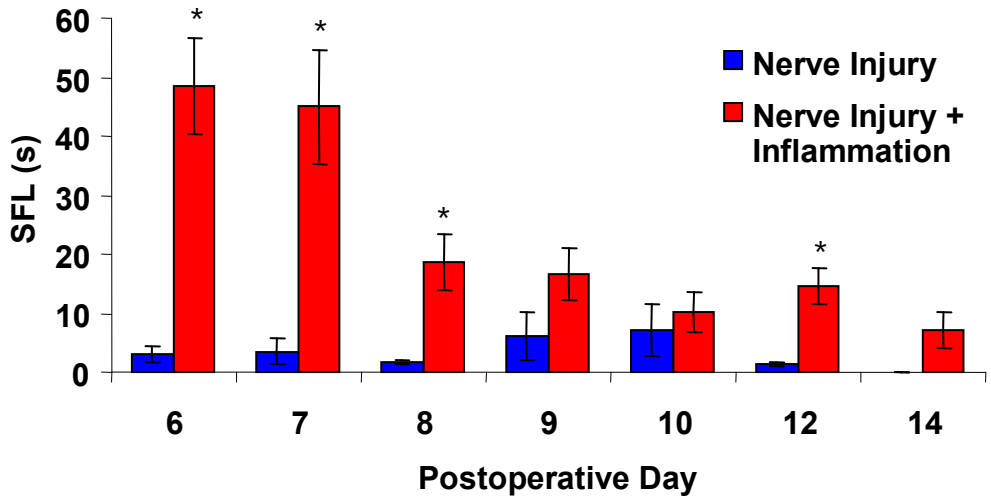


Figure 3.3. Non-evoked spontaneous foot-lifting (SFL) of the injured paw following nerve injury (■), or nerve injury + inflammation (■) in adult rats. Data are expressed as mean±SEMs for the cumulative time over 3 minutes in which the injured paw was raised. (*$p<0.05$, t-test nerve injury + inflammation compared to nerve injury alone; n=3-6). No SFL of the contralateral paw was observed and no SFL was observed in the inflammatory pain group and so is not included in this graph.

3.2.3 Increased association of αCaMKII with NR2A/B subunits at peak sensitisation following adult injury

In trial experiments, western blots of adult spinal NR2A/B immunoprecipitates (see chapter 2, 2.8) probed for αCaMKII showed an increased association of these proteins ipsilateral to injury, 10 days (during peak sensitisation) following inflammation or a combination of nerve injury and inflammation compared to contralateral (n=7 pooled samples) and also to naïve control (n=4 pooled samples). Blots were also probed for NR2B to monitor the levels of the receptor pulled-down. No immunoreactivity was detected in control lanes from non-immune immunoprecipitates. All samples were run on the same gel to allow cross comparisons between groups. It was not possible to analyse these data statistically as samples were pooled in order to carry out the co-immunoprecipitation. Data are calculated as grey scale value relative to NR2B expression (Figure 3.4). Some pilot studies were also carried out with PSD-95 immunoprecipitates, which appeared to show small increases in association of stargazin and also chapsyn-100/PSD-93 with PSD-95 10 days following inflammation (data not shown).

As the changes in αCaMKII:NR2B association were relatively modest and required substantial animal numbers to be pooled to achieve detectability, it was decided not to pursue this line of investigation in the adult, nor to extend it to neonatally injured animals.

Figure 3.4

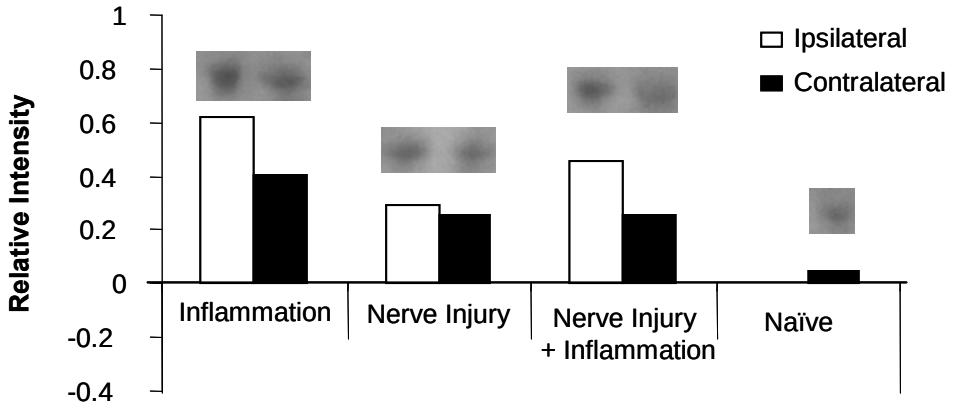


Figure 3.4. Injury-induced changes in association of αCaMKII with adult spinal NR2A/B immunoprecipitates. Data are plotted as the relative grey scale intensity (arbitrary grey scale values), relative to NR2B levels, for ipsilateral (▭) and contralateral (■) dorsal horns (n=7 pooled samples) 10 days following injury (inflammation, nerve injury, or combined nerve injury and inflammation) or in controls (naïve, n=4 pooled samples). Representative images from scanned immunoblots are displayed above the relevant data points.

3.2.4 ATF-3 expression in DRG neurons is increased following nerve injury or the combination of nerve injury plus inflammation

As ATF-3 expression in DRG cells is though to be associated with explicit injury to their axons (Tsujino et al., 2000), we assessed the expression levels of this marker in the three different adult pain models (Figure 3.5). ATF-3 positive cells from 3-4 non-adjacent sections (100μm) of L4 and L5 DRG per animal (n=6 per group) were counted, and expressed as a % of the total number of cells counted in these sections. DRG neurons showed ATF-3 expression following nerve injury or nerve injury plus inflammation, but not following inflammation alone. One-way ANOVA (Student-Newman-Keuls post-test for all-pairwise comparisons) indicated that inflammation resulted in significantly lower ATF-3 expression compared to nerve injury ($p<0.001$) and nerve injury plus inflammation ($p=0.003$). ATF-3 expression in DRG cells was not significantly different ($p=0.091$) in nerve injury compared to nerve injury plus inflammation.

Figure 3.5

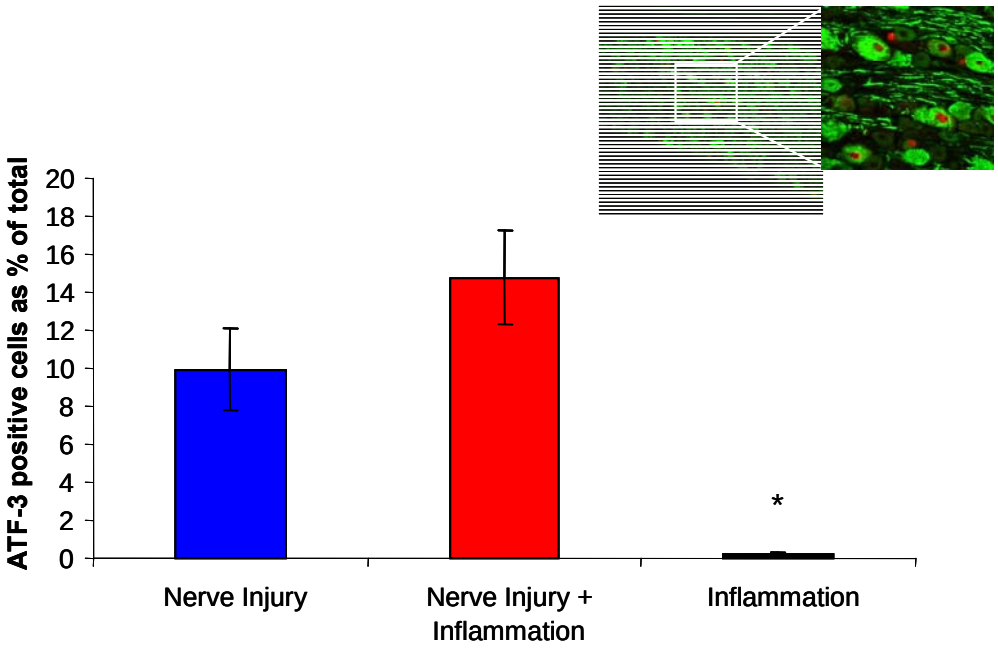


Figure 3.5. ATF-3 expression in adult-injured DRG neurons 10 days post injury (n=6). Data are expressed as a percentage of total cells counted per group for nerve injury, nerve injury plus inflammation, and inflammation. Inset shows confocal image (x10 magnification) of ATF-3 (red) colocalised with NF-200 (green), in DRG following nerve injury + inflammation. (*$p<0.05$, One-way ANOVA Student-Newman-Keuls post-test for all-pairwise comparisons, inflammation compared to both nerve injury and nerve injury and inflammation)

3.2.5 Previous nerve injury or inflammation in adults does not alter the response to a subsequent noxious challenge upon recovery

Following recovery from the sensitised state induced by injury (approx 70 days), animals were given an injection of formalin (Figure 3.6) into the plantar surface of the hindpaw, ipsilateral to the original injury (nerve injury, inflammation or combined pain model). The injection was given under brief inhalation anaesthesia to minimise stress, and as a result the first phase behavioural response is lost, as has previously been demonstrated (Fukuda et al., 2001). However the peripheral input that generates the behaviour observed in the second phase still occurs (Dickenson

and Sullivan, 1987) and, importantly, we are able to measure the nocifensive behaviour associated within this phase. The response curve we have obtained following formalin administration is similar to the classically described second phase response in terms of its magnitude and duration (Fukuda et al., 2001). The formalin response of all 3 groups of previously injured adult animals was not significantly different to that of naïve controls (two-way ANOVA).

Figure 3.6

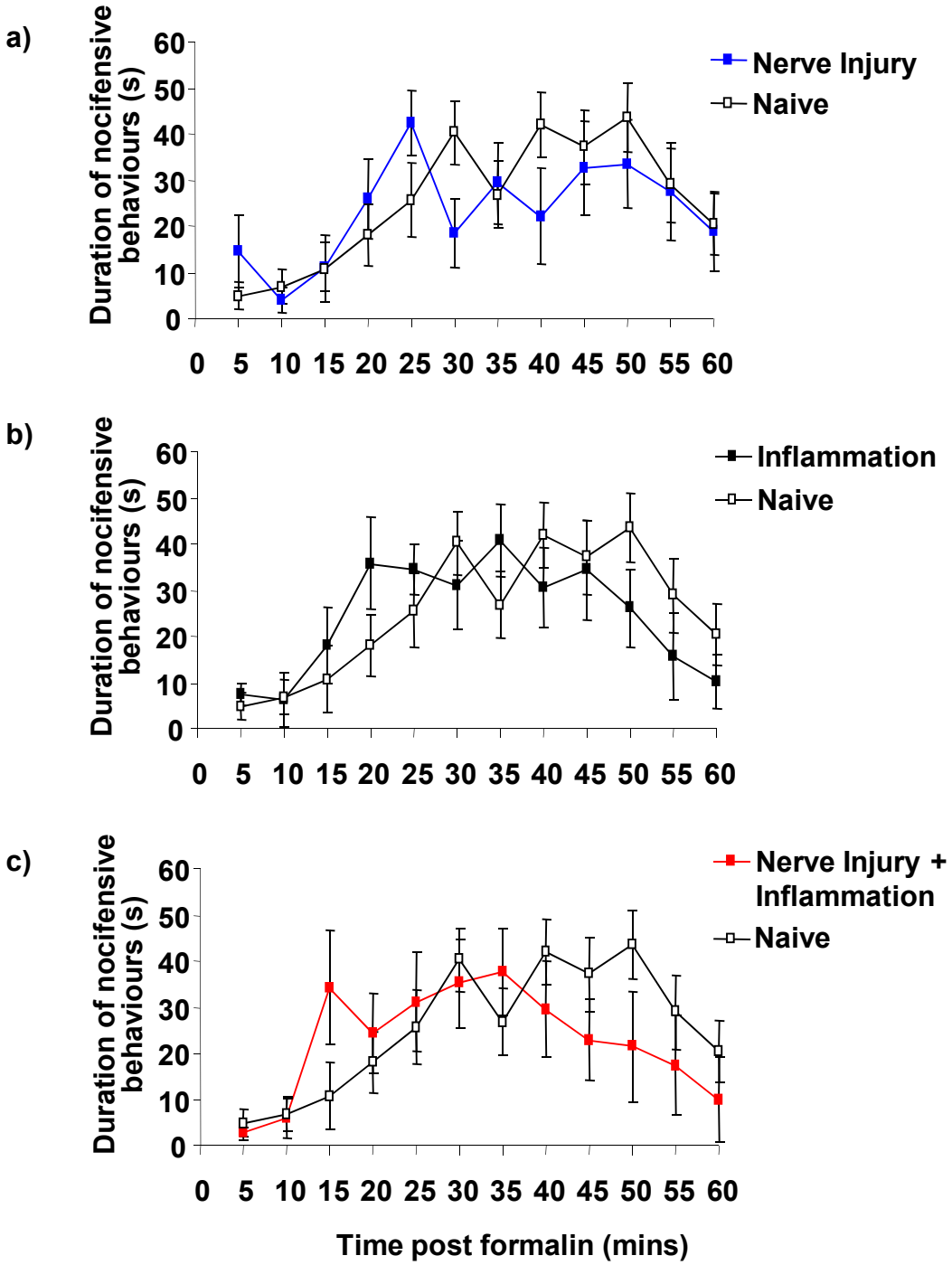


Figure 3.6. Adult response to a formalin challenge following recovery from prior injury. a) Nerve injury (▪ n=6), b) inflammation (▪ n=6) or c) a combination of these two injuries (▪ n=5), each compared to naïve controls (▫ n=8). Mean±SEM is plotted in seconds (s) for cumulative amount of nocifensive behaviour recorded per minute for each time point post-formalin injection. There were no significant differences between groups (two-way AVOVA).

3.3 Discussion

3.3.1 A combined neuropathic and inflammatory injury results in longer lasting hypersensitivity and spontaneous pain

Animal models of clinical pain are widely studied to improve our understanding of the aetiology of persisting pain states and also to identify potential therapeutic targets for the development of novel analgesics. There is a continuing need for animal models which best represent clinical conditions, both in terms of duration and also the symptoms and responses observed. The novel combined pain model was designed to encompass both inflammatory and neuropathic injuries, which often occur together (Said and Hontebeyrie-Joskowicz, 1992) either clinically or as a result of routine animal husbandry procedures. In our novel combined pain model, both evoked and non-evoked measures of pain behaviour are enhanced when peripheral inflammation accompanies nerve injury (Figures 3.1 to 3.3). In terms of evoked measures of nociceptive behaviour, it should be considered that the large difference in PWTs in the combined pain model, and indeed the single pain models, could have been attributed in part to an increased contralateral PWT due to unwillingness to bear weight on the injured ipsilateral paw. However, as contralateral PWTs do not differ over time between the groups, it seems likely that significant differences observed for changes in PWT (contralateral- ipsilateral) are due to increases in sensitivity of the injured paw and not a reduced sensitivity/unwillingness to withdraw the contralateral paw. This model provides us with a robust and long-lasting pain state which displays symptoms such as hypersensitivity to mechanical and thermal stimuli, as well as non-evoked raising of the injured paw, (spontaneous foot-lifting) which is thought to be an indicator of spontaneous pain (Bennett and Xie, 1988).

Spontaneous pain is a major clinical problem, which is not sufficiently represented by the basic science studies currently being published, where much of the sensory behaviour is focused on withdrawal responses to mechanical or thermal stimuli, when in fact 96% of neuropathic pain patients have ongoing, spontaneous pain and although still significant, only 64% and 38% have a mechanical or thermal component, respectively (Backonja and Stacey, 2004). The validity of reflex pain

behaviours, as measured using von Frey filaments and the Hargreaves' test, although useful and reproducible, has been questioned recently in favour of implementing more spontaneous pain measures, or operant evoked measures (Mogil, 2009). Such operant evoked measures consider motivational responses to noxious stimuli and may provide a way to reflect the emotionality associated with some chronic pain states which is a major problem in human subjects, who often report that symptoms can worsen following emotional upset (Backonja and Stacey, 2004). For these reasons it may have been useful to assess evoked and non-evoked measures following affective testing on the elevated plus maze and open field test for example, which were carried out to assess any affective component associated with the novel combined pain model compared to single models. Interestingly, pilot studies in these tests of affective state did not highlight any differences in the three pain models investigated compared to non-injured naïve animals (data not shown).

The possible reasons for the increased sensitivities to mechanical and thermal stimuli, and also the spontaneous pain component observed in the novel pain model have not yet been sufficiently clarified. Previous work has suggested that spontaneous pain is associated with spontaneous firing of intact C-fibres (Djouhri et al., 2006). The study describes how a spinal-nerve axotomy (L5) and a spinal-nerve axotomy combined with loose-ligation of a neighbouring (L4) spinal nerve, both produce spontaneous activity in intact nociceptive C-fibres but only the latter version is associated with spontaneous foot-lifting behaviour (SFL). The study also reports SFL following CFA, but only on day 1 after injection and not on day 4, which is consistent with our study which finds no SFL from day 6 onwards, although we have not looked at earlier time points as animals with nerve injury were allowed to recover from the muscle damage incurred as a result of surgery during this interim period. The authors reported a correlation between the rate of spontaneous firing of intact C-fibres and the observation of SFL in all three models, and suggest that inflammatory mediators may contribute to the enhanced spontaneous firing rate in the modified spinal-nerve axotomy/L4 ligation model. The findings of this study may help to explain the degree of spontaneous behaviour observed in our combined nerve injury and inflammation model. Inflammatory mediators, such as interleukins (IL) and TNFα are released during Wallerian degeneration of damaged nerves (Shamash et

al., 2002) and have been linked with spontaneous activity in DRG neurons (Schafers et al., 2003). Furthermore, the upregulation of NGF, induced in response to the increase in TNFα following peripheral inflammation (Woolf et al., 1997), has also been shown to lead to spontaneous activity in DRG neurons (Djouhri et al., 2001). Although the site of peripheral inflammation in our combined pain model is not at the nerve trunk, the combined neurogenic inflammation associated with the CCI component, and the peripheral inflammation following CFA may exert a cumulative effect, which may be required to increase the rate of spontaneous activity of nociceptive C-fibres to the levels necessarily for the generation of spontaneous behaviour.

This combined pain model may be a useful model to utilise in future basic science studies to assess the basis for the greater mechanical and thermal sensitivities induced in this model compared to single models, and perhaps more importantly to investigate the clinically-relevant spontaneous pain component. Further lines of investigation into the spontaneous component of this model would have been useful. Pharmacological studies may be helpful to dissect the major underlying mechanisms which may be responsible for the enhanced evoked and non-evoked pain behaviours.

3.3.2 Increased association of CaMKII with NR2A/B subunits in response to injury

This study has focused on the NMDA receptor and its associated proteins as a potential mechanism for sensitisation. For this reason we looked at the association of the NR2A/B complex with CaMKII, an association thought to be involved in LTP and synaptic plasticity (Barria and Malinow, 2005; Lee et al., 2009). Although we can not perform statistical analyses on the data, due to sample pooling to achieve sufficient material, the study has indicated that there is an increased αCaMKII:NR2B association following inflammation and also when inflammation and nerve injury were combined (Figure 3.4). As previously discussed (1.5.3) the NMDA receptor has been linked with injury-induced nociceptive behaviours (Mao et al., 1992; Yaksh et al., 1995; Chaplan et al., 1997) and its downstream signalling pathways, particularly the association with CaMKII have been implicated in sensitisation induced by nerve injury and inflammation (Fang et al., 2002; Garry et al., 2003). Garry et al 2003

(Garry et al., 2003) show that both autophosphorylated (constitutively activated) phospho-Thr (286) αCaMKII and total αCaMKII are upregulated ipsilateral to injury in spinal NR2A/B immunoprecipitates and that this upregulation is dependent on NMDA receptor activity. Interestingly, recent studies using mutants with a point mutation at this phosphorylation site (position 286 of the αCaMKII gene) suggest that autophosphorylated αCaMKII is important in the generation of spontaneous, non-evoked, pain (Zeitz et al., 2004), which, importantly, seems to be a differentiating feature of the combined pain model.

3.3.3 The addition of peripheral inflammation to a nerve injury in the novel combined pain model does not enhance explicit nerve damage, as revealed by ATF-3 expression

To determine the extent of neuronal damage following the combined pain model, ATF-3 expression was compared to single-injury adult pain models (Figure 3.5). ATF-3 is known to be a marker of nerve injury and is induced in models of neuropathic pain (Tsujino et al., 2000). We have shown that although there is more nociceptive sensitisation associated with the combined pain model, this is not due to a greater degree of nerve injury, as shown by similar levels of ATF-3 expression between the combined pain model and the CCI model or nerve injury. As a marker of explicit nerve injury, ATF-3 is generally not induced in response to peripheral inflammation (Palm et al., 2008; Segond von et al., 2009). We have shown that there is little or no ATF-3 expression in response to CFA, in agreement with other studies, and it is likely that the CFA component of the combined pain model does not enhance the induction of ATF-3.

3.3.4 Prior adult injury does not induce long-lasting changes in nocifensive behaviour in response to formalin

The formalin response of previously injured adult animals was not different between the groups, nor was it different to that of naïve animals (Figure 3.6). A previous study investigating the effect of giving a formalin injection two weeks following

nerve injury, shows enhanced pain associated with the formalin response (LaBuda et al., 2001). This is unsurprising given that formalin was administered relatively soon after the initial injury, when withdrawal thresholds in response to nerve injury were still likely to be low (i.e. sensitised). Conversely, a similar study using the same model of neuropathic pain (spinal nerve ligation) reported that the formalin response is *reduced* in animals four weeks after nerve injury compared to sham controls (Kaku et al., 2007). The possible reasons for these opposing results may be due to timing of formalin injection relative to the time course of the injury and also possibly due to the differing formalin concentrations used. In agreement with the latter study, Vissers et al (Vissers et al., 2003) have shown a reduction in the formalin response in both the ipsilateral and contralateral paw of animals 8 days following CCI, suggesting that the activation of supraspinal, descending inhibitory pathways may account for the reduction when there has been a prior injury. These studies highlight the importance of the timing at which formalin is given following a prior injury, as well as the nature of the specific nerve injury model used. It seems that our study is the first to address the effect of a subsequent noxious challenge upon complete behavioural recovery from the initial insult. In our work, formalin was administered under brief anaesthesia, upon recovery from the initial injury, to minimise stress, but it might have been useful to have carried out the formalin challenge without anaesthesia, thus leaving the first phase intact. This phase is thought to reflect acute nociception due to the activation of nociceptors on primary afferents, and although our data represent an intact and robust second phase due to the initial nociceptor activation, it might be interesting to assess the first phase following a prior injury, as it is highly likely that similar acute challenges would occur clinically in chronic patients.

3.4 Conclusions

We have introduced a novel model of chronic pain which combines both neuropathic and inflammatory events which are clinically relevant occurrences. This model may prove to be a useful tool in investigating the mechanisms of the clinically relevant spontaneous pain behaviour, as well as the robust mechanical allodynia and thermal

hyperalgesia associated with this model and this may potentially lead to the identification of new therapeutic targets.

CHAPTER 4: PAIN BEHAVIOUR IN THE EARLY POSTNATAL PERIOD

4.1 Introduction

In the adult, the plasticity of the nervous system allows for the development of long-lasting sensitised states following experimental nerve injuries such as CCI (Bennett and Xie, 1988) and also in inflammatory pain models. In the neonate however, the impact of similar injuries is unclear, as the nervous system continues to develop new connections and is undergoing constant reorganization. This may mean that the neonatal nervous system is able to compensate in some way to certain insults, or that long-lasting deleterious changes are put in place. There is a need for further investigation of the long-term effects of invasive procedures such as those that may occur clinically in preterm human infants and also in the previously unexplored tail-docked piglet. Previous studies have shown varying long-term effects of early life injury. It has been reported that peripheral nerve injury does not induce sensitisation when carried out in young animals aged 3-21 days, and adult-like allodynia is not induced until P33 (Howard et al., 2005). However, other studies have indicated that the neonatal nervous system is capable of generating nociceptive responses in response to nerve injury within the first two postnatal weeks (Lee and Chung, 1996; Back et al., 2008). There seems to be less confusion as to the degree to which young animals are able to respond to an inflammatory insult. Some studies have reported sensitisation in response to rather large doses of CFA, in the first postnatal week, with adverse long-term effects upon re-inflammation later in life (Ruda et al., 2000; Tachibana et al., 2001). Similar responses have been observed with another well characterised, shorter-lasting inflammatory agent, carrageenan (Lidow et al., 2001).

4.2 Results

4.2.1 Sustained mechanical allodynia is induced following nerve injury at P8 but only transiently following peripheral inflammation

Mechanical allodynia between P8 injured males and females was compared in each model and found not to be different (not shown); for this reason all data discussed, unless otherwise stated, refer to groups containing both males and females. Nerve injury, or a combination of nerve injury and inflammation, at P8 induced sensitivity to mechanical stimuli that was significant for 21 days post injury (Figure 4.1, $p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral paw). It was not possible to begin nociceptive reflex testing until around 10 days post injury (i.e., P18), and therefore pre-surgery baselines are not possible, as animals are too small and immature at this point. Furthermore there is evidence that reflex behavioural responses to stimuli do not become fine tuned until after P10, and gradually improve thereafter, becoming adult-like and appropriately scaled to the applied stimuli by around P20 (Holmberg and Schouenborg, 1996; Waldenstrom et al., 2003). In response to inflammation at P8, there was a delayed and transient change in PWT, which was significant at 35 days post injury ($p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral paw) despite an increase in oedema (Figure 4.2) in the injured paw, which significantly increased the dorsoventral distance of the injured paw until post-injury day 7 (** $p<0.01$, *$p<0.05$, t-test, comparing ipsilateral to contralateral); a shorter-lasting response than that which occurs in the adult, which is maintained up to day 20 (Figure 4.2, $p<0.05$ at day 20).

In addition to the relatively short-lasting sensitivity (for 3 weeks post-injury) observed following nerve injury (nerve injury alone or in combination with inflammation), we also found that a subset of animals remained sensitive to mechanical stimuli for much longer (greater than 21 days) (Figure 4.3), up to 105 days following nerve injury, and 70 days in the combined pain model ($p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral paw). It should be noted that day 87 in the combined pain model (Figure 4.3b) fails to reach significance despite an obvious difference in ipsilateral/contralateral PWT indicated

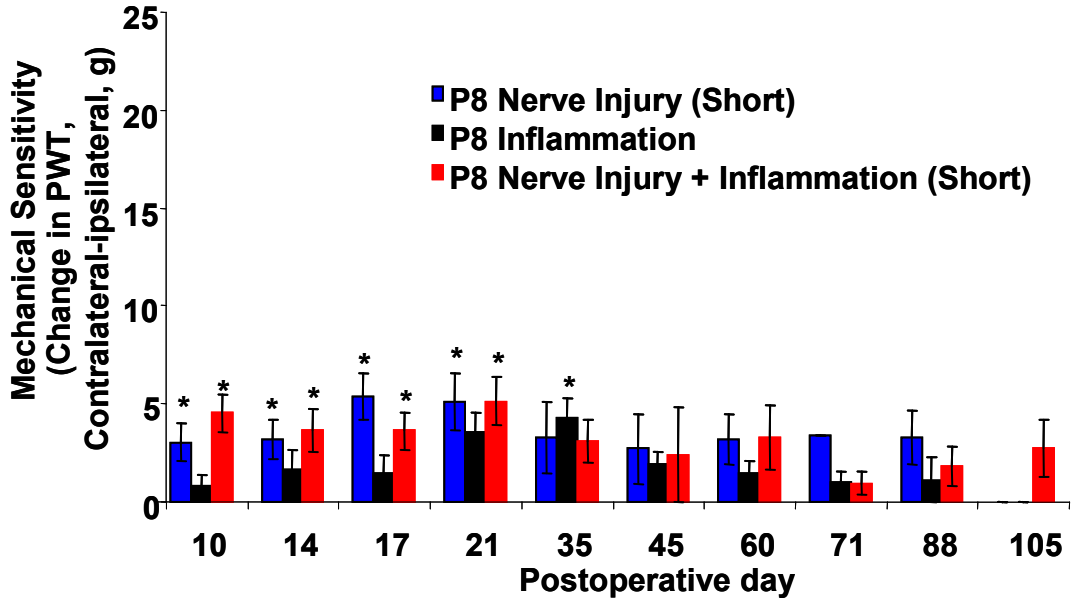
on the graph, and this may be due to a low number (n=3) of animals at this point. Additionally, day 105 in the long-lasting combined pain model is just below the level for significance (Figure 4.3b, p=0.057). Two-way ANOVA revealed that the curves representing the time-course for long-lasting or short-lasting mechanical allodynia (Figure 4.3) are significantly different from one another for both nerve injury (Figure 4.3a, $F=29.43$, $p<0.0001$) and also for the combined pain model ($F=47.95$, $p<0.0001$, with significance over time indicated from day 35 onwards, $p=0.0003$). We also carried out light microscopy analysis of semi-thin sections of sciatic nerves from animals that had long or short-lasting responses to nerve injury (both n=3) or from naïve animals (n=2) and found no significant difference in *G*-ratios between the groups (data not shown).

As a combined pain model at P8 does not appear to be different, in terms of the degree and duration of mechanical sensitivity, to nerve injury alone (as it is in the adult), it was decided that future studies would focus on only the nerve injury alone or inflammation alone, in order to refine experiments and reduce the number of animals used. Control animals were anaesthetised and removed from their home cage for the same duration as injured animals and mechanical PWTs were tested. There were no differences between the PWT of the hindpaws in anaesthetic controls, and PWTs for both paws increases with development, as expected (Holmberg and Schouenborg, 1996).

Animals were weighed regularly to identify any signs of distress. Weight gain was not different between naive animals and injured animals with both long and short-lasting mechanical allodynia (data not shown).

Figure 4.1

a)



b)

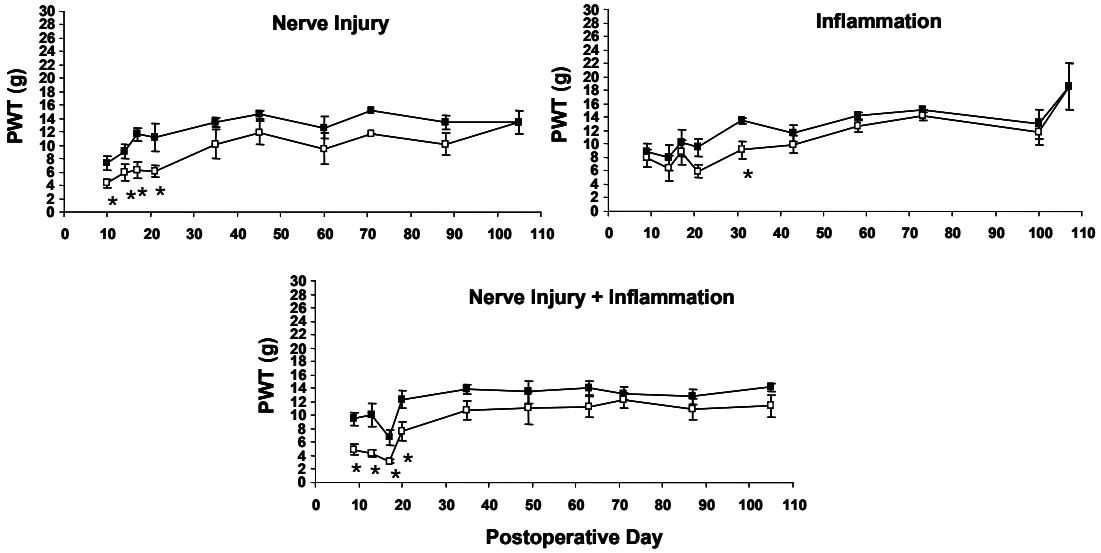


Figure 4.1. Time course of mechanical allodynia following P8 injury. Data are expressed as mean±SEMs of a) the difference in paw withdrawal threshold (PWT) following P8 nerve injury (■), P8 inflammation (■) or a combination of these two injuries (■) or b) as absolute values for ipsilateral (open squares) and contralateral (filled squares) paws. Data are obtained from calibrated von Frey filaments (1.2-28.8g) Day of surgery is day 0. (*$p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral paw; n=6-13 days 10-45 n=3-4 days 60-105)

Figure 4.2

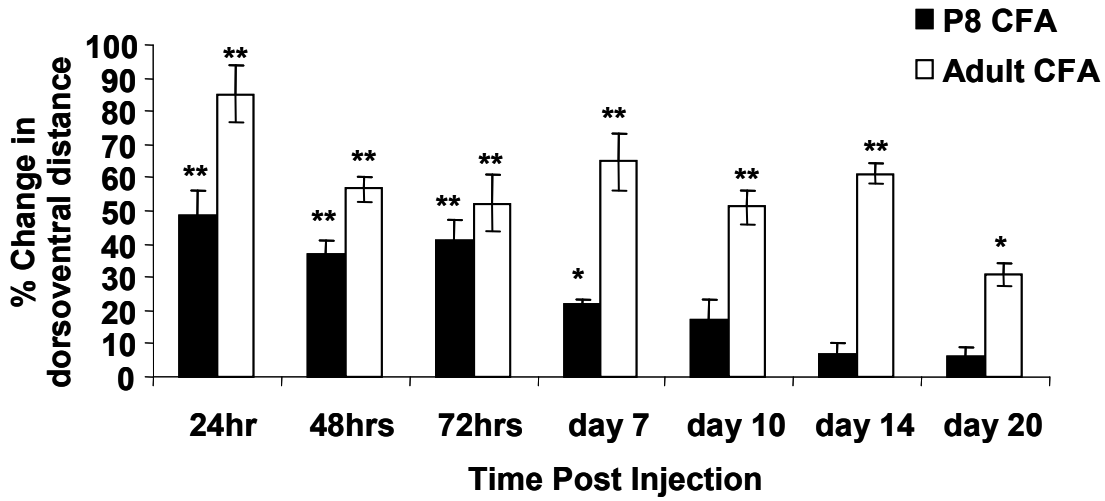


Figure 4.2. Inflammation induced changes in dorsoventral distance of the ipsilateral hindpaw (compared to contralateral), in adult (□) and P8 injured animals (■) post CFA injection (** $p<0.01$, *$p<0.05$, t-test, comparing ipsilateral to contralateral; n=3-4).

Figure 4.3

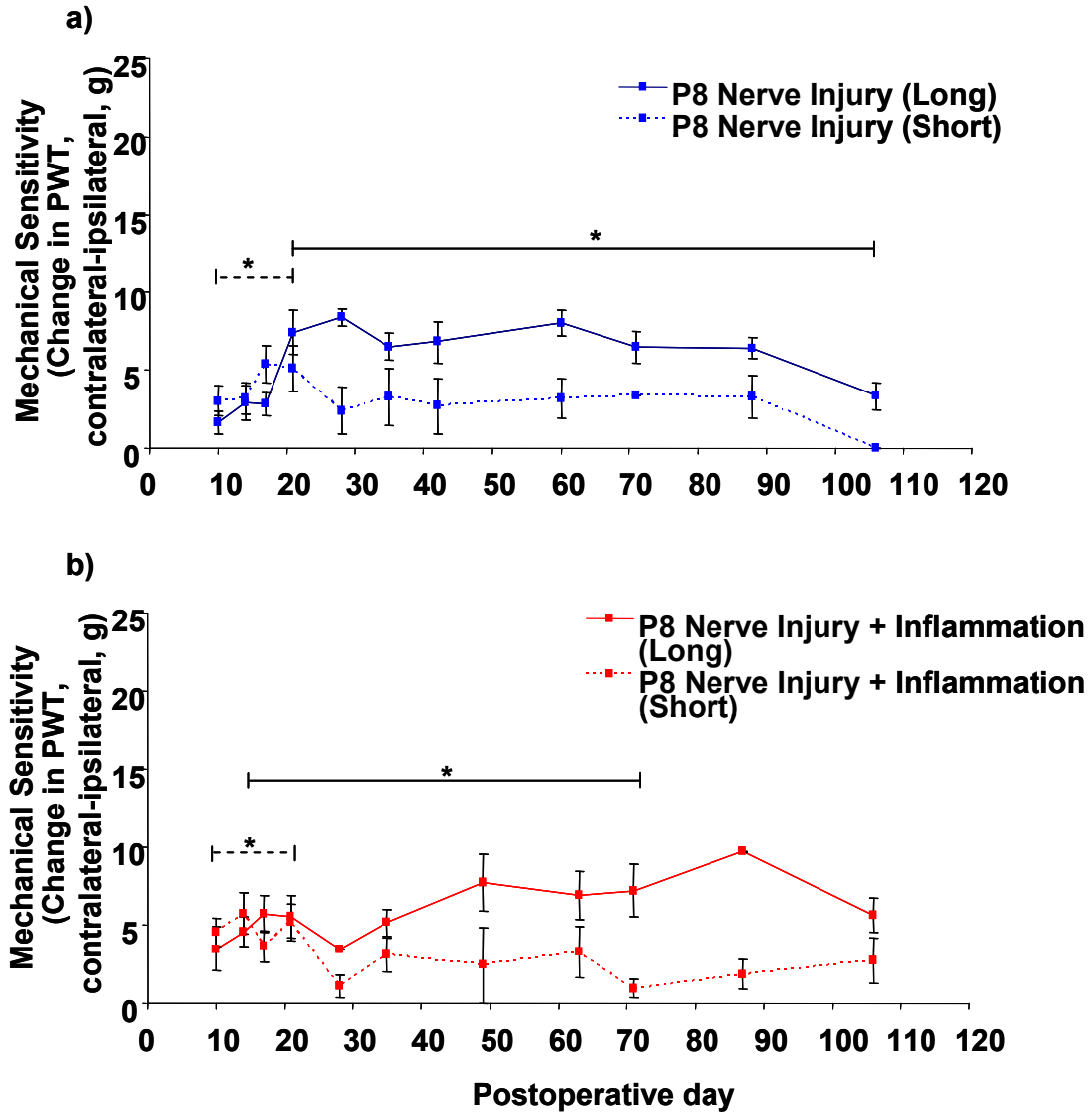


Figure 4.3. Differing durations of sensitisation of behavioural reflex responses to mechanical stimuli following a) P8 nerve injury, or b) a combination of nerve injury and inflammation to compare the long (solid line) and short (dotted line)-lasting variations of the injuries. Long-lasting sensitisation was defined as that lasting longer than 21 days. Day of surgery is day 0. Data are expressed as mean±SEMs of the difference in paw withdrawal threshold (PWT), contralateral-ipsilateral, from calibrated von Frey filaments (1.2-28.8g) (*$p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral paw; n=3-13).

4.2.2 The time at which nerve injury occurs in the neonatal period is crucial in determining whether mechanical allodynia is elicited

Interestingly, when nerve injury was performed two days later than P8, at P10, we found that the degree of sensitisation achieved did not reach significance (Figure 4.4). This experiment was carried out to compare our data with a study that appears to contradict our finding that neonatal nerve injury can induce sensitisation (Howard et al., 2005). We have shown that we are able to replicate the finding of that study, which shows that P10 CCI does not induce mechanical allodynia (Figure 4.4). These data therefore provide evidence that the precise age at which neonatal injury is carried out may be crucial. It should, however, be noted that this preliminary study on the effects of P10 nerve injury was carried out using one whole litter (n=12), whereas P8 and P18 studies were carried out on animals from a range of different litters. It is therefore possible that the lack of effect seen following P10 injury may be due to subtle differences existing between litters. It would therefore be useful to repeat this study using pups from a range of different litters to confirm this finding.

We have also carried out these injuries at P18 in some pilot studies. At this age, responses were intermediate between those observed in the adult and those at P8 (Figure 4.5). Mechanical allodynia was induced in all three models at P18, and the combined pain model displayed higher levels of sensitisation, although some points did not reach significance, which is probably due to low numbers of animals.

Figure 4.4

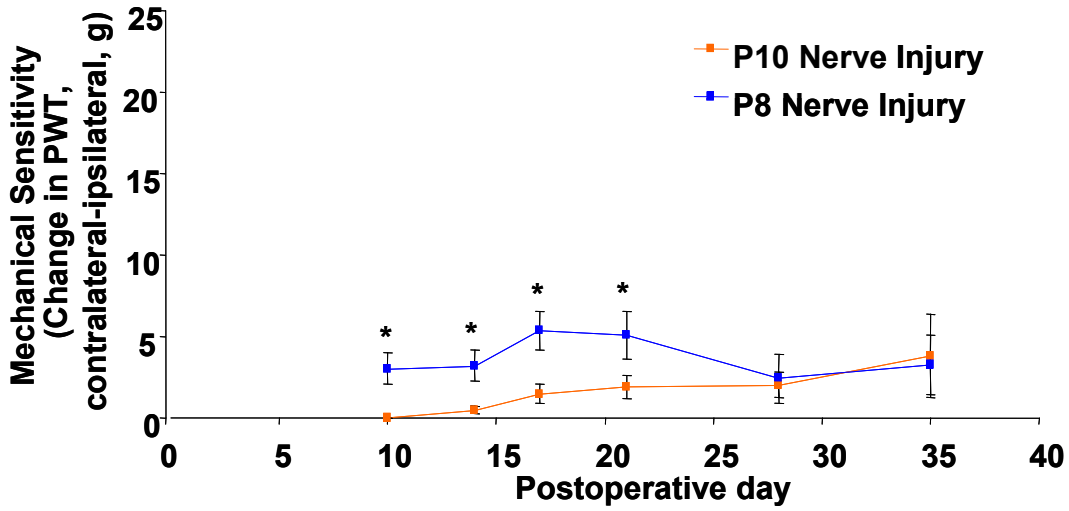
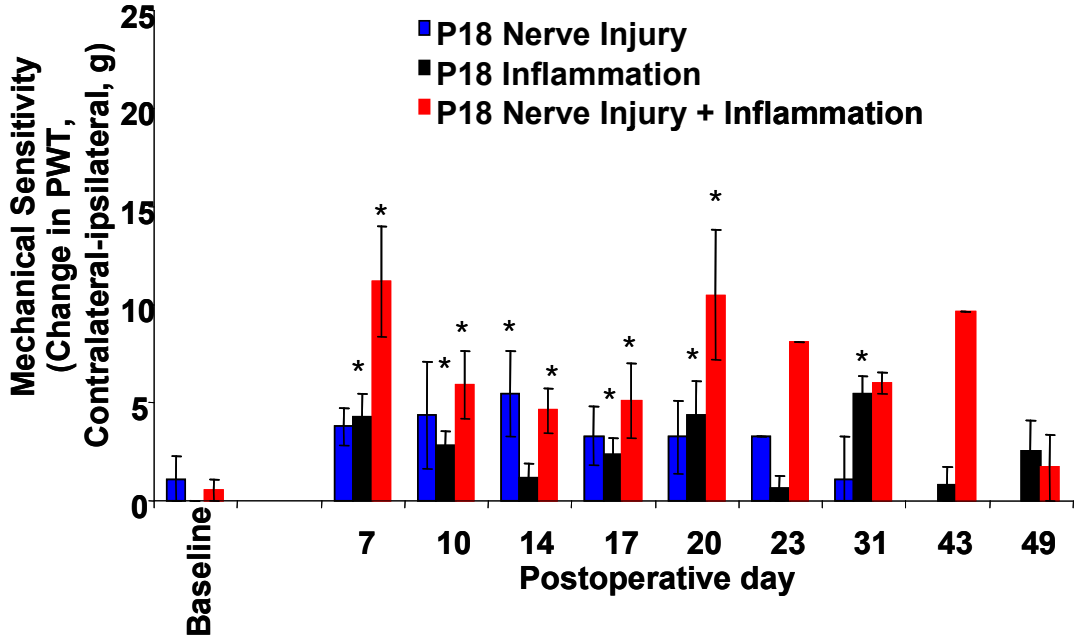


Figure 4.4. Time course of mechanical allodynia following P8 nerve injury (■), or P10 nerve injury (▪). Day of surgery is day 0. Data are expressed as mean±SEMs of the difference in paw withdrawal threshold (PWT), contralateral-ipsilateral, from calibrated von Frey filaments (1.2-28.8g) (*$p<0.05$ Mann-Whitney Rank Sum Test, P8 nerve injury, ipsilateral compared to contralateral paw; n=3-12)

Figure 4.5

a)



b)

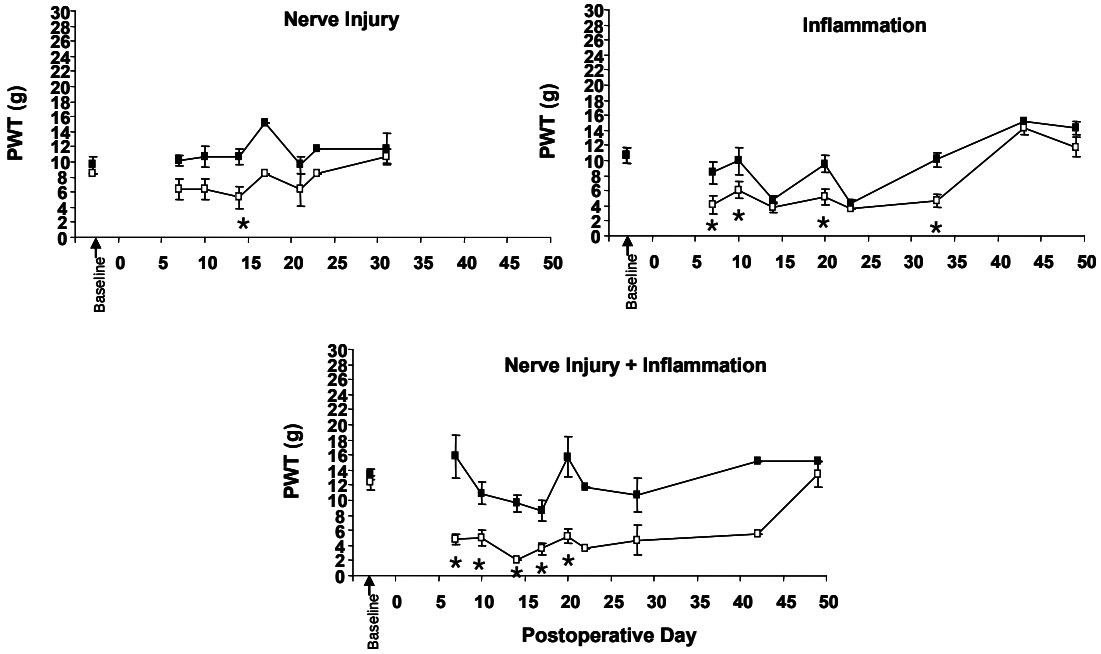


Figure 4.5. Time course of mechanical allodynia following P18 injury. Data are expressed as mean±SEMs of a) the difference in paw withdrawal threshold (PWT) following P18 nerve injury (■), P18 inflammation (■) or a combination of these two injuries (■) or b) as absolute values for ipsilateral (open squares) and contralateral (filled squares) paws. Data are obtained from calibrated von Frey filaments (1.2-28.8g) Day of surgery is day 0. (*$p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral paw; n=2-10).

4.2.3 *Injury at P8 induces greater ATF-3 expression in DRG neurons compared to adult injury*

ATF-3 expression in DRG cells is though to be associated with explicit injury to their axons (Tsujino et al., 2000). We have assessed expression levels following adult injury (Chapter 3, Figure 3.6) and also compared this to that of P8-injured animals (Figure 4.6). ATF-3 positive cells from 3-4 non-adjacent (100µm) sections of L4 and L5 DRG per animal (n=6 per group) were counted, and expressed as a % of the total number of cells counted in these sections. The proportion of cells showing ATF-3 expression in DRG neurons was significantly higher (t-test) in all 3 pain models 10 days after injury in P8-injured animals compared to adult injury. As observed in adult-injured neurons, ATF-3 expression remained extremely low in response to peripheral inflammation compared to models involving nerve injury.

Figure 4.6

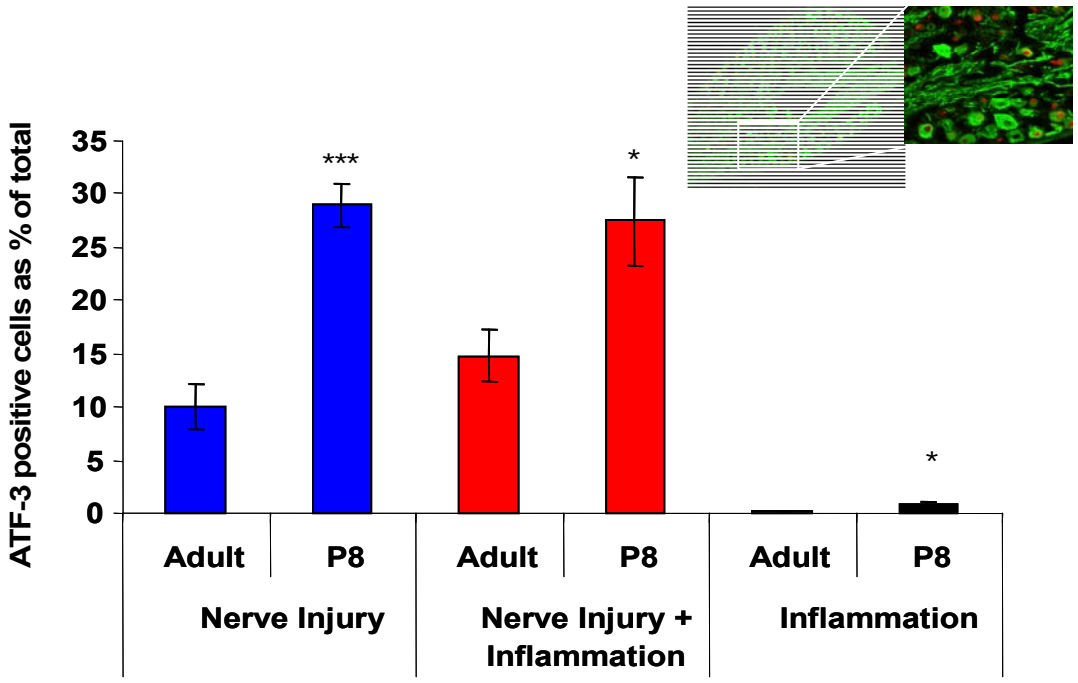


Figure 4.6. ATF-3 expression in DRG neurons 10 days post adult or P8 injury. Data are expressed as a percentage of total cells counted per group for nerve injury (■), nerve injury plus inflammation (■), and inflammation (■). Inset shows confocal image (x10 magnification) of ATF-3 (red) colocalised with NF-200 (green), in DRG following nerve injury + inflammation. (*p<0.05, ***p<0.0001, t-test comparing adult to P8; n=6).

4.2.4 Mechanical allodynia in response to P8 nerve injury is reversed following intrathecal administration of the NMDA antagonist (R)-CPP

Mechanical allodynia was reversed, without any motor impairment, following intrathecal administration of the NMDA receptor antagonist (R)-CPP. Careful dose titration to determine the optimum dose in P28 animals revealed that the NMDA antagonist (R)-CPP (1.2nmol) was able to reverse mechanical allodynia (Figure 4.7 a) 20 days after P8 nerve injury (during peak sensitisation). The ipsilateral reversal was statistically significant at 15-35 minutes post-intrathecal injection (n=6), as revealed by one-way repeated measures ANOVA on ranks with Dunn's post test to compare to pre-drug, baseline. Saline (vehicle) injection (Figure 4.6 b) of an equivalent volume (20µl), did not alter the PWT of the ipsilateral hindpaw (n=5).

Trials were also carried out on a separate set of animals using the AMPA receptor antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), however it proved difficult to determine an appropriate dose with the limited numbers of animals available and so these trials were abandoned.

Figure 4.7

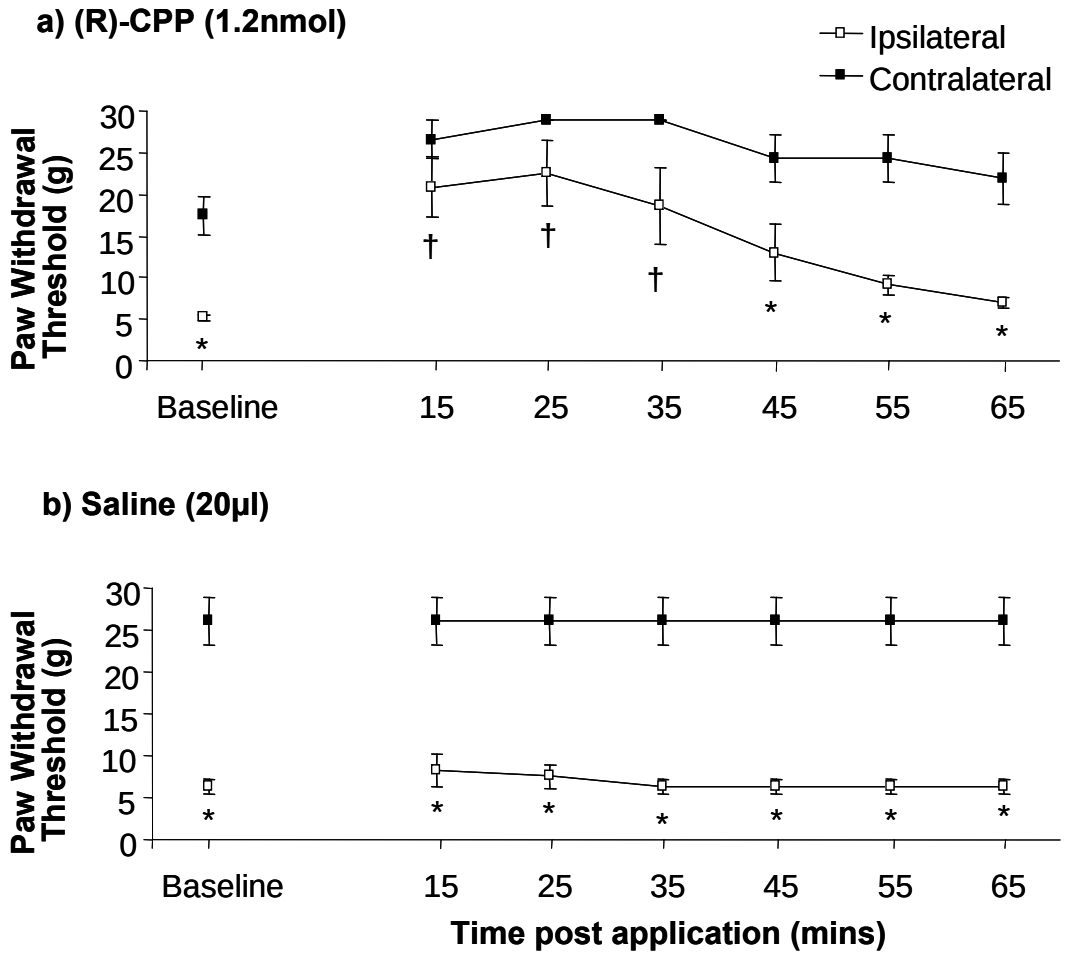


Figure 4.7 The effect of intrathecal application of a) (R)-CPP (20µl, n=6) or b) saline (n=5) at 20 days post P8 nerve injury (P28 males). Data are expressed as mean PWT±SEM from calibrated von Frey filaments (1.2-28.8g) for baselines and from 15-65 minutes post-intrathecal injection for ipsilateral (▫) and contralateral (▪) paws (*$p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral. † $p<0.001$, One-way repeated measures ANOVA on Ranks with Dunn's post test to compare post injection ipsilateral to baseline).

4.2.5 Key spinal cord proteins that are thought to be involved in the sensitisation associated with adult pain states are developmentally regulated

The NR2B NMDAR subunit and its associated proteins in the spinal cord are developmentally regulated (Figure 4.8). Our data show that NR2B expression decreases with development from P3 to P10 (n=3), and is present at low levels at P18

and P28 (n=1). αCaMKII expression remains low from P3 to P10 (n=3) but is increased by P18 and P28 (n=1). PSD-95 appeared to increase slightly from P3 to P6 but expression remained relatively unaltered from P3 to P28 (n=1-3). Data are plotted as the relative grey scale intensity (arbitrary grey scale values), relative to levels of GAPDH. It was not possible to investigate the NR2A NMDAR subunit and its associated proteins in the time available although this will be carried out for future publication.

Figure 4.8

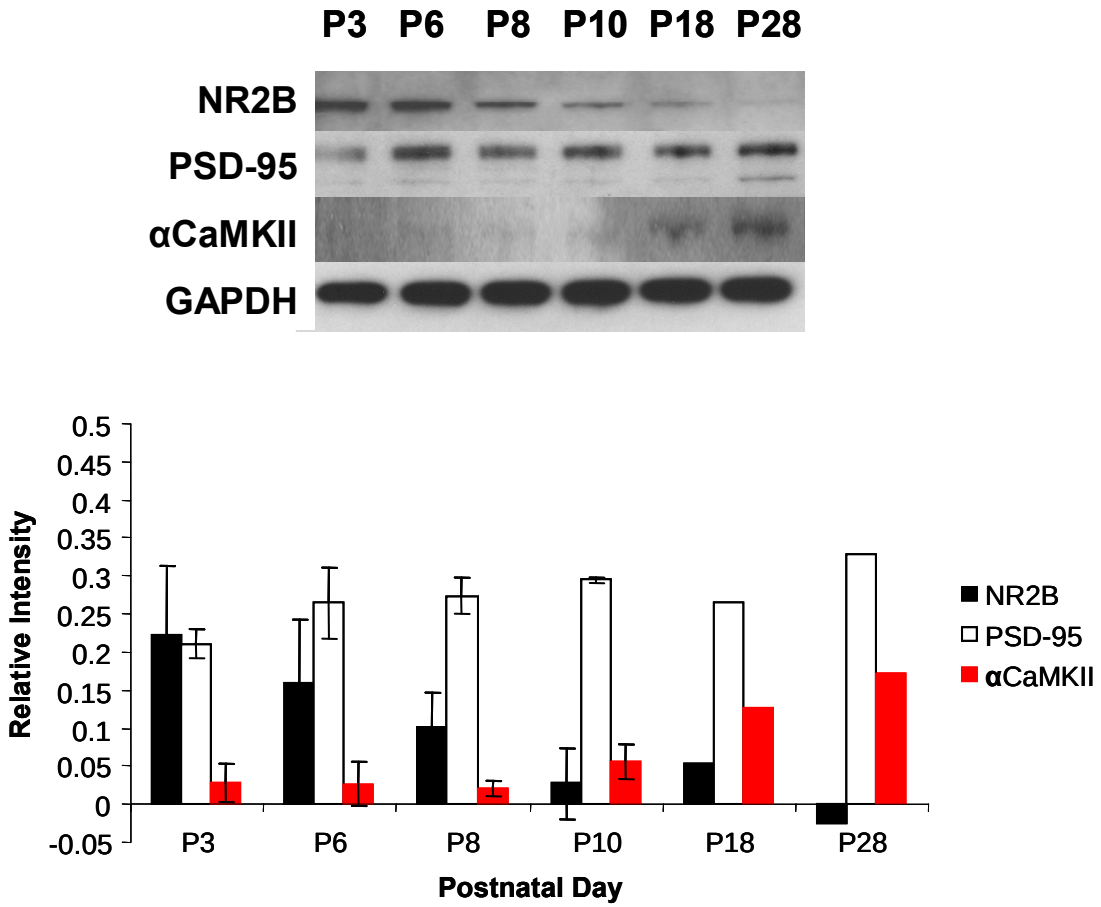


Figure 4.8. Developmental changes in the spinal cord expression of the NR2B NMDAR subunit and its associated proteins. NR2B expression decreased with development from P3 to P10 (n=3). PSD-95 expression appeared relatively low at P3 and increased by a small amount from P3 to P28 (n=1-3). The low level of PSD-95 shown here at P3 was not typical and was partially compensated for by relatively low GAPDH levels at P3. αCaMKII remained low from P3 to P10 (n=3) but was increased by P18 and P28 (n=1). Data are plotted as the relative grey scale intensity (arbitrary grey scale values), relative to GAPDH levels.

4.2.6 P8 nerve injury enhances nocifensive responses to formalin at P42 but not at P68

Although mechanical allodynia, measured using von Frey filaments was not different in young males compared to females, the response to formalin was found to be different and therefore male and female responses have been analysed separately. We have also investigated the effects of handling and maternal separation as a distinct postnatal event by comparing the formalin response of animals anaesthetised and tested using von Frey filaments at the same time as injured animals, to the response of naive/undisturbed animals. We found that in both males and females, there is no difference in the nocifensive responses of handled animals compared to naives, and we have therefore used naive animals as the control group in subsequent formalin tests to compare against responses of previously injured animals.

The formalin response at P42 was enhanced in female animals that had nerve injury at P8 (n=10) compared to age-matched naives (n=9) (Figure 4.9, c). The enhancement in nocifensive behaviour was significant ($p<0.05$) at 25 and 35 minutes post formalin injection, which represents peak second phase responses, that are thought to reflect central sensitisation induced by afferent drive from the first phase (two-way ANOVA with Bonferroni post test). There were no significant differences observed in the formalin responses of male animals following P8 inflammation (n=8) or nerve injury (n=7) (Figure 4.9 b and d, respectively) compared to controls (n=15) or between females following P8 inflammation (n=7) compared to naïve (Figure 4.9 a). There are no significant differences when analysing area under the curve data at different stages of the second phase formalin response (Figure 4.9 e and f)..

When formalin was administered at P68 (Figure 4.10), in animals that had received nerve injury at P8, the overall time course was significantly different in both male ($p<0.0001$) and females ($p=0.0259$) when compared to age matched naives (two-way ANOVA with Bonferroni post test), although no individual time points were significantly different.

Figure 4.9

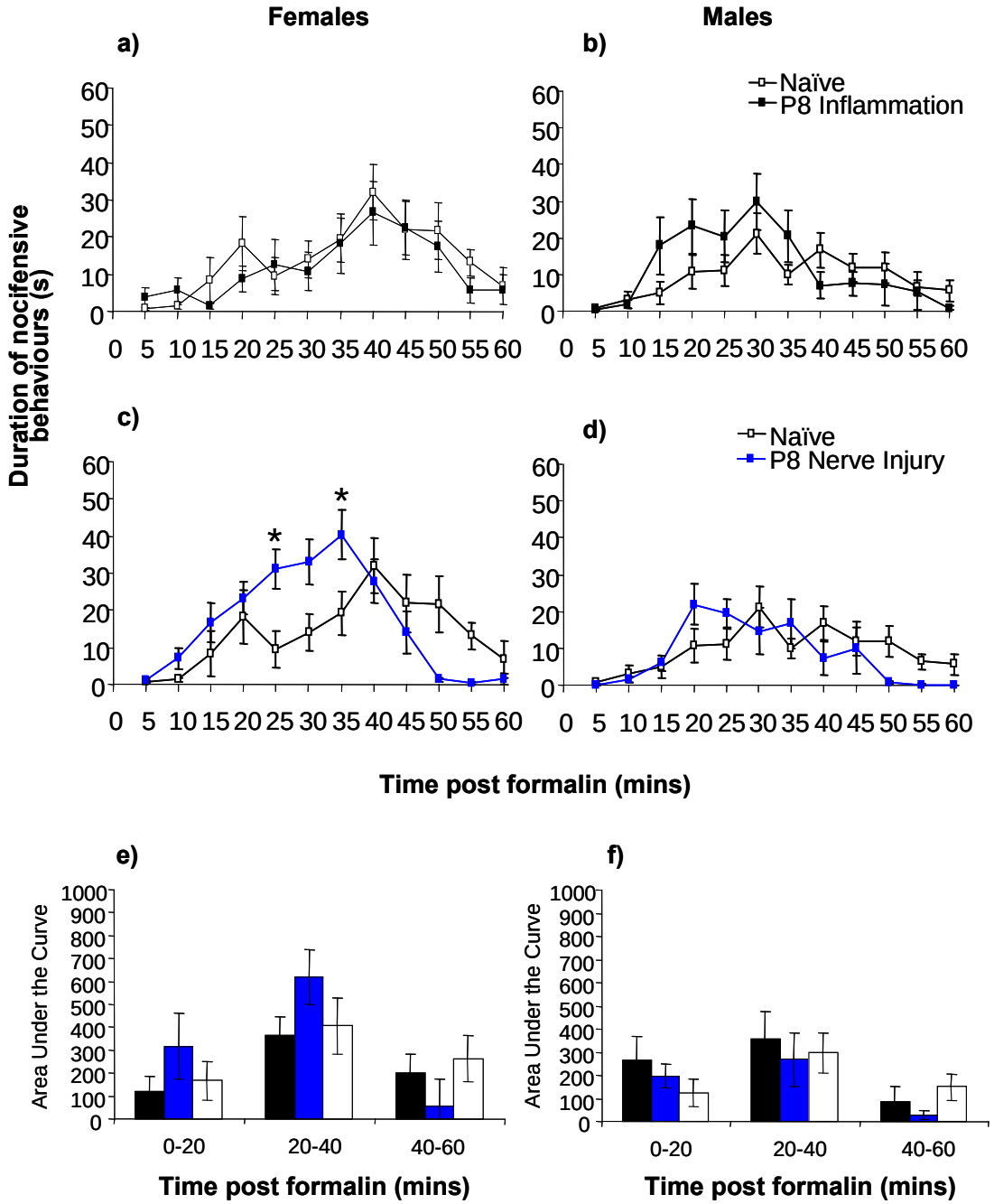


Figure 4.9. P42 female (left, a, c and e) and male (right, b a, d and f) nocifensive responses to a formalin challenge following recovery from prior injury at P8. Nerve injury (▪) or inflammation (▪) are compared to naïve controls (▫) (a-d) and area under the curve is shown for females (e) and males (f) at early (0-20 minutes) peak (20-40 minutes) and late (40-60 minutes) stages of the second phase of the formalin response. Mean±SEM are plotted in seconds (s) for cumulative amount of nocifensive behaviour recorded per minute for each time point (a-d) post-formalin injection. Area under the curve (e and f) was calculated using the trapezoidal rule for different satges of the second phase formalin response. (*p<0.05 two-way ANOVA with Bonferroni post test; n=7-15).

Figure 4.10

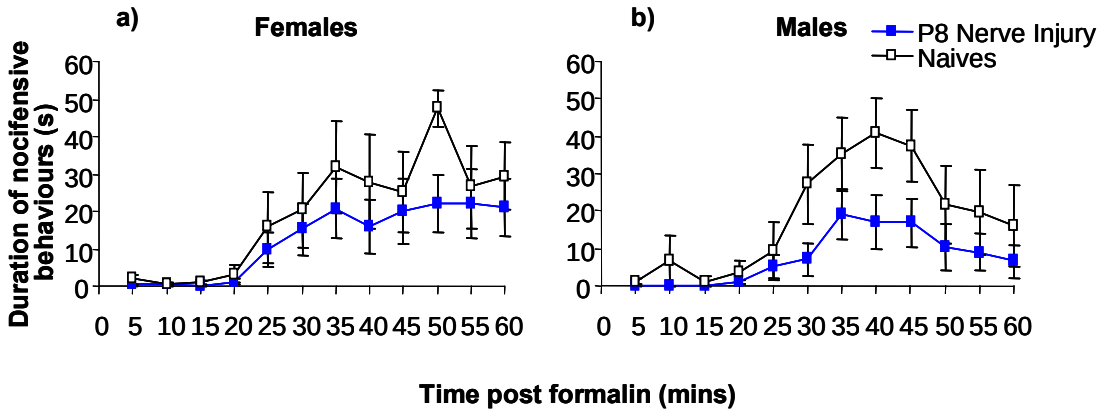


Figure 4.10. P68 female and male responses to a formalin challenge following recovery from prior injury at P8. Nerve injury (n=9▪) compared to naïve controls (n=5▫). Mean±SEM is plotted in seconds (s) for cumulative amount of nocifensive behaviour recorded per minute for each time point post-formalin injection. Two-way ANOVA with Bonferroni post-tests showed that there was a significant overall effect of treatment in a) $p=0.0259$ and b) $p<0.0001$, suggesting that the formalin response over time is significantly lower following a prior P8 injury than in naive controls.

4.3 Discussion

4.3.1 Long-lasting sensitisation to mechanical stimuli can be induced in the neonatal period but is age and injury specific

There is currently some confusion regarding the consequences of neonatal injuries such as nerve injury or inflammation in the rodent. Some studies have reported an absence of any behavioural sensitisation in response to peripheral nerve injury (Howard et al., 2005), whereas others have reported that nerve injury is able to induce short-lasting sensitisation from as early as first postnatal week (Lee and Chung, 1996). Similarly, the degree to which peripheral inflammation in the neonatal period is able to alter nociceptive processing is unclear and seems to be dependent on the developmental age and inflammatory agent used, with some studies reporting sensitisation as well as altered nociceptive responses to noxious stimuli in later life (Ruda et al., 2000; Tachibana et al., 2001; Lidow et al., 2001). Additionally there is similar discrepancy over the consequences of neonatal injuries in human patients (Johnston and Stevens, 1996; Taddio et al., 1997; Whitfield and Grunau, 2000). It seems that there is no clear answer as to the way in which nociceptive processing can be altered in response to any given neonatal injury and it is dependent on the timing and type or injury incurred (Lidow, 2002).

Our results suggest that the age and type of injury carried out is crucial in determining whether or not mechanical allodynia can be induced (Figure 4.1-4.5). We have shown that it is possible to induce mechanical allodynia in response to nerve injury carried out at P8 (Figures 4.1 and 4.3), but not when this injury is carried out at P10 (Figure 4.4). We have carried out P10 CCI to corroborate our finding that neonatal nerve injury is able to induce mechanical allodynia, in the face of findings from other studies which report that early life nerve injury fails to induce sensitisation at P10 (Howard et al., 2005). Howard et al (Howard et al., 2005) reported that both CCI and SNI are unable to induce mechanical allodynia when carried out at P10, furthermore, they show that SNI within the first 3 postnatal weeks does not lead to mechanical allodynia. However, although this study is partly in agreement with our data, which suggests that P10 CCI does not induce mechanical

allodynia (Figure 4.4), the authors also fail to observe any sensitisation in response to SNI over a range of ages up to P33, whereas we have shown that nerve injury at both P8 and P18 is able to induce allodynia (Figure 4.5). There are however, some differences in the experimental procedures in that study compared to our own, mainly the SNI model is different to the CCI model that we have used, but also sensory testing methods are different. Howard et al carry out their assessments of mechanical allodynia using von Frey filaments applied to the dorsal surface of the hindpaw, presumably whilst restraining these animals, whereas we have tested the glabrous, plantar surface of the hindpaw, and these two testing methods have been shown to have slightly differing outcomes (Kauppila et al., 1998). Furthermore, P8 injuries were carried out to include animals from a number of different litters, were as our assessments of P10 nerve injured animals were carried out on animals from within a single litter. It is therefore important that this study is extended to include animals from a range of litters to rule out the possibility of inter litter variability being responsible for the observed discrepancies between injury at P8 and P10. Nevertheless, our preliminary results indicate that we may have uncovered a developmental window at P8 when it is possible to induce allodynia. Although it seems perplexing that it is possible to have two differing outcomes when the same injury is carried out at different times within a 48 hour window, the changes occurring within developing nociceptive circuitry during this time are so dynamic that it is possible that the state of the nervous system at P8 is such that long-lasting changes can be induced. Major pathways to mature after these developmental time points include the descending inhibitory pathways which are not developed until the second postnatal week (Galineau et al., 2004). It is possible that molecular and cellular responses to nerve injury that may occur in response to the increased afferent drive induced by P8 nerve injury are suppressed by the presence of newly developed inhibitory pathways that are in place by P10.

In addition to the nerve-injured animals which recover by approximately 21 days post injury, we also find that a subset of animals remain hypersensitive for much longer (Figure 4.3). Although the magnitude of sensitisation reached is similar between the peak of the short-lasting subset and that reached in the long-lasting group, sensitisation in the long-lasting group plateaus at this level and is maintained

for over 100 days. Interestingly, in addition to the duration of mechanical allodynia, we also observe that the majority of animals with long-lasting mechanical allodynia also develop abnormal posture of the injured paw, to a noticeably greater extent than that which normally accompanies peripheral nerve injury in adults (Bennett and Xie, 1988). CCI is known to produce motor disturbances in the adult (Bennett and Xie, 1988), which is thought to be due to motor denervation (Daemen et al., 1998). The tibial branch of the sciatic nerve innervates the gastrocnemius muscle which is responsible for plantar flexion of the hindpaw, and it is this particular movement is diminished ipsilateral to injury the long-lasting P8 CCI subset. This may suggest that more damage to the tibial nerve has occurred in this subset of animals, possibly due to variation in the tightness of the ligation at the time of surgery and also following surgery and throughout growth. Furthermore, as the injury and resulting nerve damage occurs just before these animals become more mobile and begin to walk it is possible that the damage incurred makes it difficult to correct for this and animals compensate by dragging the injured paw behind, and fail to bear much weight on this paw, a factor which has been shown to result in lower thresholds following assessment of mechanical allodynia (Kauppila et al., 1998). Interestingly, such motor disturbances and apparent longer-lasting hypersensitivity to mechanical stimuli are not induced following injury at P18 (Figure 4.5), when animals have already begun walking.

As previously mentioned, the inflammation model at P8 induced only delayed and transient mechanical allodynia (Figure 4.1), despite the induction of oedema in response to the CFA injection (Figure 4.2), although this was relatively short in duration compared to that observed in the adult. This is in agreement with other studies that fail to show mechanical allodynia following similar doses of CFA in the first postnatal week, even in the presence of swelling of the injured paw (Walker et al., 2003). There are however some studies that report longer-lasting consequences of neonatal inflammation (Ruda et al., 2000), however such responses were a result of relatively large doses of CFA, that would be equivalent to approximately 3x the standard adult dose (1µl/g body weight). As there was little mechanical allodynia in response to inflammation alone, it is reasonable to assume that this is the reason that the combined pain model at P8 does not display the enhanced mechanical allodynia

compared to nerve injury alone that we see in the adult. In addition, we have shown as in the adult, that neonatal inflammation does not induce ATF-3 expression in DRG, confirming the expected lack of injury to axons of sensory afferents.

We have shown that as well as being able to induce behavioural sensitisation, nerve injury at P8 also causes explicit nerve damage, as revealed by ATF-3 expression, which is significantly greater than that induced in adult-injured DRG neurons (Figure 4.6). It may be perceived that the extent of damage incurred to neonatally injured DRG neurons is greater than that in adult neurons based on this expression profile, and this damage may subsequently translate to alterations in nociceptive processing in response to noxious stimuli within the dermatomes of the damaged nerve in later life. However, Tsujino et al (Tsujino et al., 2000) reported that time course of ATF-3 expression is dependent on the distance between injury site and the DRG. It is therefore also possible that the greater induction of ATF-3 expression 10 days following neonatal injury compared to the same duration after adult injury is simply because the distance between the injury site and the DRG is smaller in the neonate. To further address this it would be helpful in future studies to determine the time course of ATF-3 expression in both adult and neonatally nerve injured animals to compare the magnitude of expression over a range of ages and times post injury. Similarly, it would be useful to compare ATF-3 expression between the long and short-lasting P8 nerve-injured animals over a number of time points to identify whether or not there is a greater extent of nerve damage in those animals where sensitisation seems to persist for greater than 4 weeks.

4.3.2 Mechanical allodynia induced by P8 nerve injury is dependent on NMDA receptor activity

We have shown that, similar to adult nerve injuries (Wilson et al., 2005), the sensitisation to mechanical stimuli following P8 nerve injury is dependent on NMDA receptor activity (Figure 4.7). This result suggests that the underlying mechanisms responsible for mechanical allodynia are similar in adults and neonates. However, we have also shown that spinal proteins involved in NMDA receptor signalling are developmentally regulated and are present at very different levels at the time of

neonatal injury compared to adult levels (Figure 4.8). Interestingly, the levels of these proteins, particularly αCaMKII, begin to reach adult levels at around the time that we see peak sensitisation (P28). We have further shown that an increased amount of αCaMKII is associated with NR2A/B complexes ipsilateral to injury following adult pain states (Chapter 3, Figure 3.5). It may be that sensitisation is only possible when αCaMKII reaches adult-like levels, and can then potentiate postsynaptic signalling within spinal circuits in response to NMDA receptor activation, which had been suggested as a mechanism for LTP and central sensitisation (Bayer et al., 2001; Ji et al., 2003). Our current data suggest that NMDA receptor signalling is crucial in maintaining mechanical allodynia in P8 injured animals, although further investigation is required in order to determine the specific mechanisms responsible, and in particular the involvement of αCaMKII ipsilateral to injury, in a similar way to that used to assess changes following adult injuries.

4.3.3 Nerve injury in early life results in an alteration in the response to formalin upon behavioural recovery from the initial insult

As discussed in chapter 3 (3.3.4) the nocifensive response to the formalin test seems to be dependent on the timing at which formalin was given following the initial injury. Although in our studies, all animals had fully recovered in terms of PWT in response to mechanical stimuli before formalin was given at P42 (Figure 4.9) or at P68 (Figure 4.10), we cannot rule out the possibility that animals may still have been sensitised to other modalities such as thermal stimuli, which would imply altered nociceptive processing and therefore a greater impact of formalin in an already sensitive animal. As P42 is closer to the end of mechanical allodynia than P68, it is possible that the enhanced response observed at 25- 35 minutes in P42 nerve-injured females (Figure 4.9 c) is due to some degree of residual sensitisation. It has been reported that female rats are more susceptible to long-lasting changes in sensory processing compared to males, which may potentially explain the observed enhancement in females and not males (LaPrairie and Murphy, 2007).Area under the curve analysis of the data (Figure 4.9 e) suggests that the differences between naive and P8 nerve-injured animals are due to a leftward shift in the formalin response in

animals with a prior nerve injury, rather than an overall enhancement in nocifensive behaviour. Similar leftward shifted stimulus/response curves to those observed for female P8 nerve-injured animals (Figure 4.9 c) have been shown in other studies following inflammatory insults where long-lasting changes in sensory circuits have been reported (Ruda et al., 2000). The authors suggested that the shift may be due to an earlier onset of nociceptor activation and/or a reduction in the descending inhibition which usually occurs between the first and second phase of the formalin response. It is possible that P8-injured females are relatively more susceptible to changes in sensory processing induced by nerve injury and that these changes persist until later life to cause this shift in the formalin response compared to controls. Further investigations with greater numbers of animals are needed to identify the pathways responsible for this apparently enhanced signalling during the earlier part of the second phase of the response to a formalin challenge.

4.4 Conclusions

Our data show for the first time that early-life nerve injury, within a very specific developmental window at P8 but not P10 in neonates is able to induce NMDA-dependent mechanical allodynia. Furthermore, we have also shown that such injuries are able induce lasting alterations to nociceptive processing, particularly in females, as revealed by altered responses to a formalin challenge in later life. Further investigations into the specific mechanisms responsible for these changes will provide a greater insight into the capabilities of the immature nervous system to respond to noxious insults and how such interactions may result in long lasting consequences. A greater understanding of these processes should help to inform clinicians of the consequences of such injuries and potential measures which could be put in place to prevent long-lasting or even permanent alterations to nociceptive processing.

CHAPTER 5: PRENATAL STRESS AND POSTNATAL PAIN

5.1 Introduction

Elevation of maternal corticosteroids is thought to be one of the mechanisms by which prenatal stress can adversely affect the HPA axis responsiveness of offspring. However, with respect to the impact of prenatal stress on postnatal pain, the role of maternal glucocorticoids on the development of nociceptive systems and their associated connections is complicated and little understood. Butkevich et al (Butkevich et al., 2009) have shown that removing maternal corticosteroids (adrenalectomy prior to mating) does not seem to affect the nocifensive response to formalin observed in prenatally stressed offspring compared to offspring from non-adrenalectomized, intact dams. The authors suggest that maternal serotonergic systems may be important in prenatal programming of pain behaviour, as previous studies from this group have shown that a reduction of maternal and fetal serotonin during critical windows of serotonergic development (P11-12) is able to significantly increase the intensity of the second phase formalin response in adult prenatally stressed male and female offspring when compared to offspring from intact stressed dams (Butkevich et al., 2005). It is difficult to determine exactly how serotonin depletion in gestation might affect offspring pain behaviour due to its many roles in stress and pain processing. Serotonin is able to modulate HPA axis responsiveness via projections from the raphe nuclei of the midbrain which act upon serotonin 2C receptors (5-HT(2C)Rs) present in CRH-containing neurons in the PVN (Heisler et al., 2007) and the last trimester is the most critical period for adverse effects of prenatal stress on the developing serotonergic system (Peters, 1988). Furthermore serotonergic neurons have direct inhibitory and excitatory descending connections with nociceptive pathways in the dorsal horn, that begin to develop in the last trimester and are not adult-like until the third postnatal week (Rajaofetra et al., 1989). Studies on prenatally stressed neonatal rats (P7) show that the formalin response is elevated compared to non-stressed controls. Additionally the formalin response in prenatally stressed rats is composed of a clear first phase, interphase and second phase whereas controls display a prolonged continuous response (Butkevich et al., 2006), as the biphasic response to formalin does not usually develop until the

second postnatal week, around the time that descending serotonergic pathways are developing (Barr, 1998). It is suggested that a decrease in descending inhibition and increased monoaminergic facilitation as well as a change in the developmental time course of these processes may occur with prenatal stress, to help explain these alterations to the formalin response (Butkevich et al., 2006). Interestingly, spinal GABAergic neurons have been shown to mature earlier following a reduction of 5-HT (Allain et al., 2005), which can occur following prenatal stress (Huizink et al., 2004).

In addition to its effects on descending pathways, prenatal stress may also alter nociceptive processing more directly, at the level of the spinal dorsal horn. Studies have shown that central GRs are involved in the upregulation of NMDA receptors following nerve injury, and that blockade of spinal GRs by intrathecal antagonists or antisense oligonucleotides prevents nerve injury-induced upregulation of NMDARs and can reduce associated pain behaviour, while the GR agonist, dexamethasone is able to exacerbate neuropathic pain (Wang et al., 2005). In light of this study, it is possible to speculate that the basal and stress-induced increases in glucocorticoids observed in prenatally stressed animals may affect the development of neuropathic or other NMDA associated pain states or may even affect the postnatal development of NMDARs in the spinal dorsal horn.

The impact of prenatal stress on postnatal pain has not yet been studied extensively. The effect of the combination of these two adverse events in the perinatal period has received very limited attention and given the likelihood of both perinatal stress and pain occurring together in both animal husbandry practices and also in pre-term human patients where these two events are not always mutually exclusive, it is imperative that this area is more widely studied. The formalin challenge can be used as both a measure of nociceptive responsiveness and also as a stressor to measure the HPA axis responsiveness of an animal (Taylor et al., 1998) and as such we have utilised this test in this study to achieve measures of stress and pain responsiveness from a single cohort of animals and thus refining animal usage.

The present work uses the resident-intruder paradigm as a model of prenatal stress which has a strong psychological component as well as a physical component, in the event of attack. This social stress model is more ethologically relevant, when

compared to some of the above mentioned models, not only with respect to husbandry practices, which are, in part, the focus of this thesis, but also in the type of stress experienced by pregnant women, in the form of domestic violence, which can affect a worryingly large proportion of pregnant women worldwide (Cook and Bewley, 2008).

5.2 Results

5.2.1 Prenatal stress does not affect the mechanical allodynia induced as a result of P8 or adult nerve injury

In adult male nerve-injured animals, or animals with the combined nerve injury and inflammation we observed no difference in the mechanical thresholds of the plantar surface of the hindpaw of compared to non-stressed controls (Figure 5.1 a and c). However prenatally stressed (PNS) animals with adult inflammation appeared to display higher levels of mechanical allodynia over time compared to non-stressed injured animals (Figure 5.1 b) which was significantly different at day 6 post injury (two-way ANOVA with Bonferroni post test, $F=9.14$, $p=0.0036$). There was no difference in the mechanical thresholds of the plantar surface of the hindpaw in females compared to males with the combined nerve injury and inflammation, nor was there any difference due to the addition of prenatal stress in either of these groups (data not shown).

Mechanical allodynia following P8 nerve injury (Figure 5.2 a) or inflammation (Figure 5.2 b) was not altered in prenatally stressed animals compared to controls (two-way ANOVA), although the days when significant sensitisation occurred in response to P8 inflammation were slightly earlier in prenatally stressed animals compared to controls.

It is worth noting that the failure to reach statistically significant mechanical allodynia in prenatally stressed and nerve injured models (Figure 5.1 and 5.2 a) is probably due to low numbers of prenatally stressed animals available for these tests, but it is important to note that the time course does not differ compared to the non-stressed nerve injured models. The inevitable variability incurred with nerve injury

models makes it necessary to include a larger number of animals in order to reach significance. This is not the case to such an extent in the inflammatory pain models where a known volume of inflammatory agent can be reproducibly administered within a small area of the plantar surface of the hindpaw.

Figure 5.1

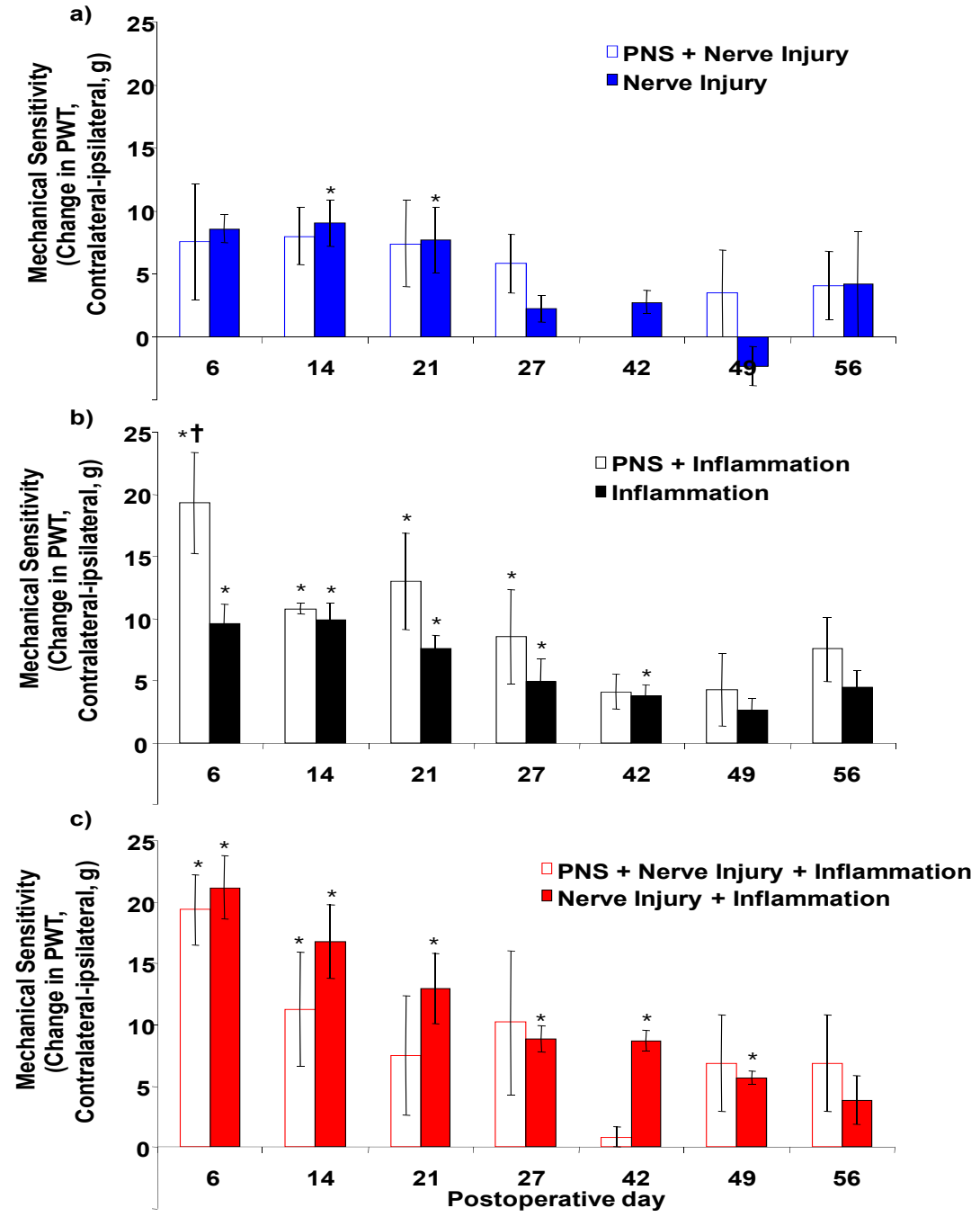


Figure 5.1 Time course of mechanical allodynia following adult a) nerve injury (■), b) inflammation (■) or c) a combination of these two injuries (■) in prenatally stressed (PNS, open bars) male animals compared to non-stressed animals (solid bars). Day of surgery is day 0. Data are expressed as mean±SEMs of the difference in paw withdrawal threshold (PWT), contralateral-ipsilateral, from calibrated von Frey filaments (1.2-28.8g) (†$p<0.05$ two-way ANOVA with Bonferroni post test, PNS compared to control group. *$p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral paw; n=4-6).

Figure 5.2

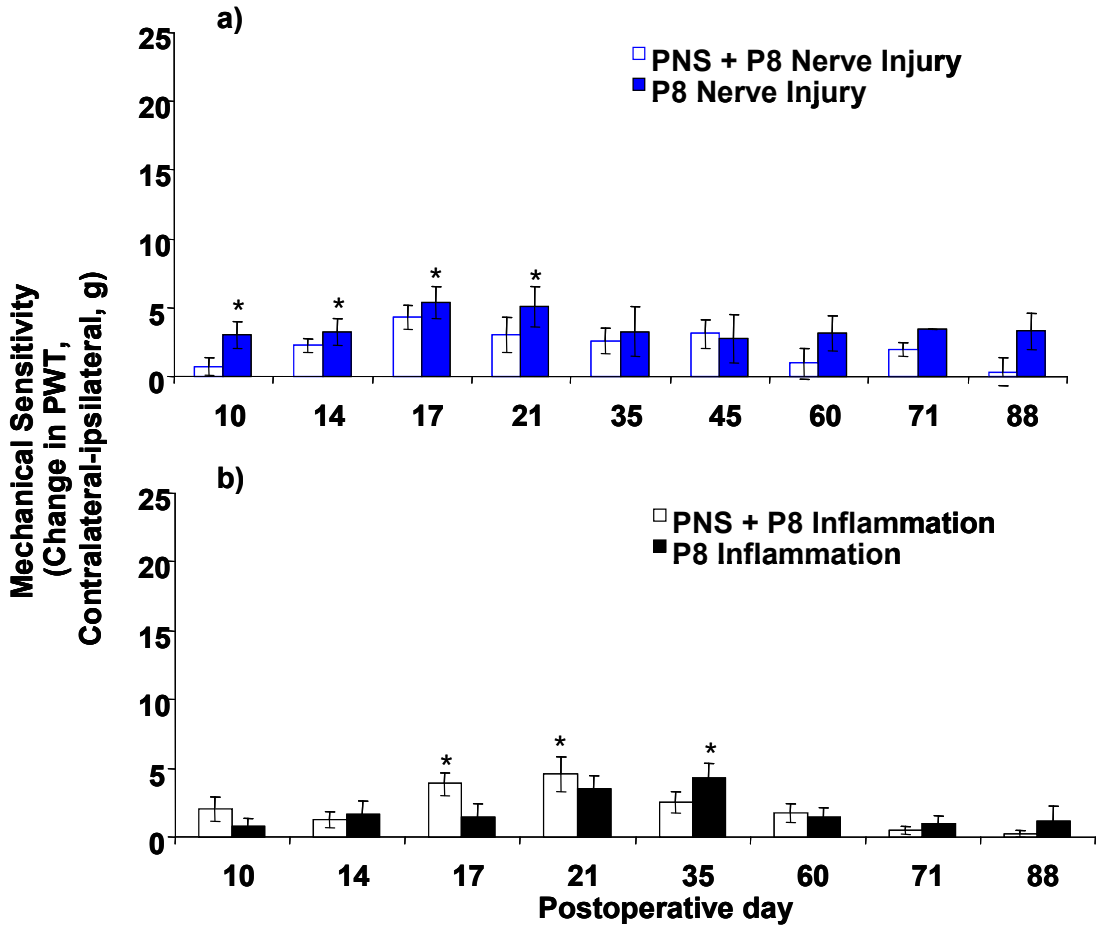


Figure 5.2. Time course of mechanical allodynia following P8 a) nerve injury (short-lasting sub-group ■), or b) inflammation (■) in prenatally stressed (PNS, open bars) animals compared to non-stressed animals (solid bars). Day of surgery is day 0. Data are expressed as mean±SEMs of the difference in paw withdrawal threshold (PWT), contralateral-ipsilateral, from calibrated von Frey filaments (1.2-28.8g) (*$p<0.05$ Mann-Whitney Rank Sum Test, ipsilateral compared to contralateral paw; n=4-12)

5.2.2 Prenatal stress does not affect the adult formalin response following recovery from adult injury but enhances the response in animals which had inflammation at P8

Prenatally stressed animals differed in their nocifensive responses to a formalin challenge depending on the age at which they received the initial insult. The formalin response of prenatally stressed males (n=3-6) which were injured in adulthood was not significantly different (two-way ANOVA) from non-stressed, adult injured animals (Figure 5.3).

Adult males which had prenatal stress along with early life injury, in the form of P8 inflammation (n=5), displayed significantly greater ($F=3.43$, $p=0.0177$), early responses in the time course of nocifensive behaviour in response to formalin (Figure 5.4) when compared to naïve (n=10) males (two-way ANOVA with Bonferroni post test). Specifically, at the 25 minute time point post-formalin, nocifensive responses in prenatally stressed, P8 inflamed animals were significantly greater than those of naïve controls ($p<0.05$). It should be noted that the number of animals in the non-stressed, P8 inflammation group was low (n=3) and increasing these numbers may also reveal differences comparing this group to the prenatally stressed, P8 inflamed group as there seemed to be an indication of differences between the curves with the current data, which would further strengthen the suggestion of an additive affect of prenatal stress (Figure 5.4). Additionally, the time course of the formalin response of prenatally stressed naïve animals also seemed to be shifted leftward slightly compared to the non-stressed, control naives, which might indicate an effect of prenatal stress alone on the formalin response, although again, this did not reach statistical significance in the present study.

There were no significant effects of treatment in prenatally stressed and P8 nerve-injured animals compared to controls (data not shown). Prenatally stressed females were also included in this study, as they were available as prenatally stressed littermates, although it is known that nociceptive responses may be affected by the phase of the estrous cycle (Kayser et al., 1996). As these animals were cycling at the time of the formalin response, swabs were taken to determine estrous which are still to be defined at a later date.

Figure 5.3

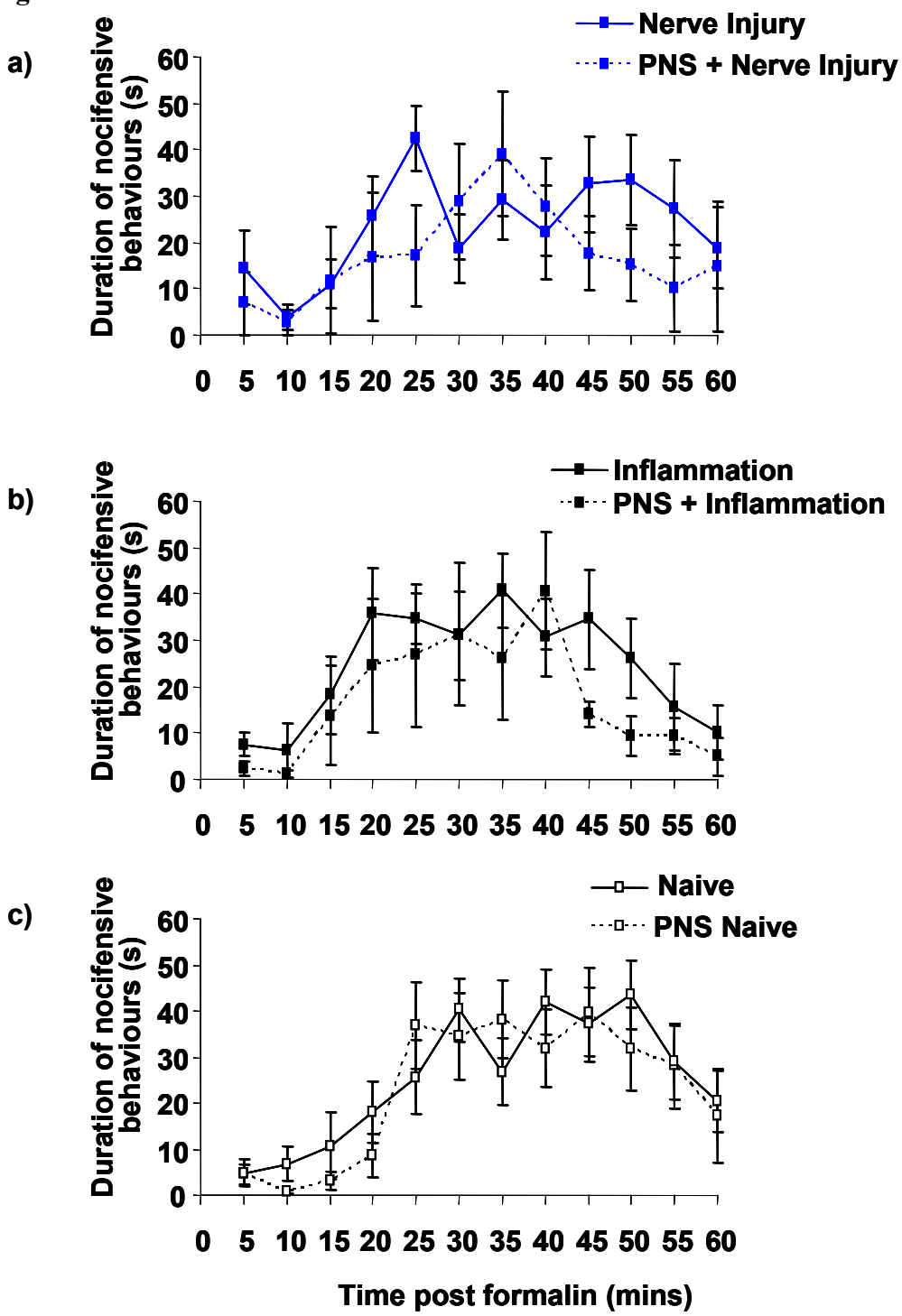


Figure 5.3. Adult male response to a formalin challenge in prenatally stressed animals (PNS dotted lines) compared to non-stressed animals (solid lines) following recovery from a) adult nerve injury (▪ n=3-5), b) adult inflammation (▪ n=4-6) or in uninjured (naive) animals (▫ n=6-8). Mean±SEM is plotted in seconds (s) for cumulative amount of nocifensive behaviour recorded per minute for each time point post-formalin injection.

Figure 5.4

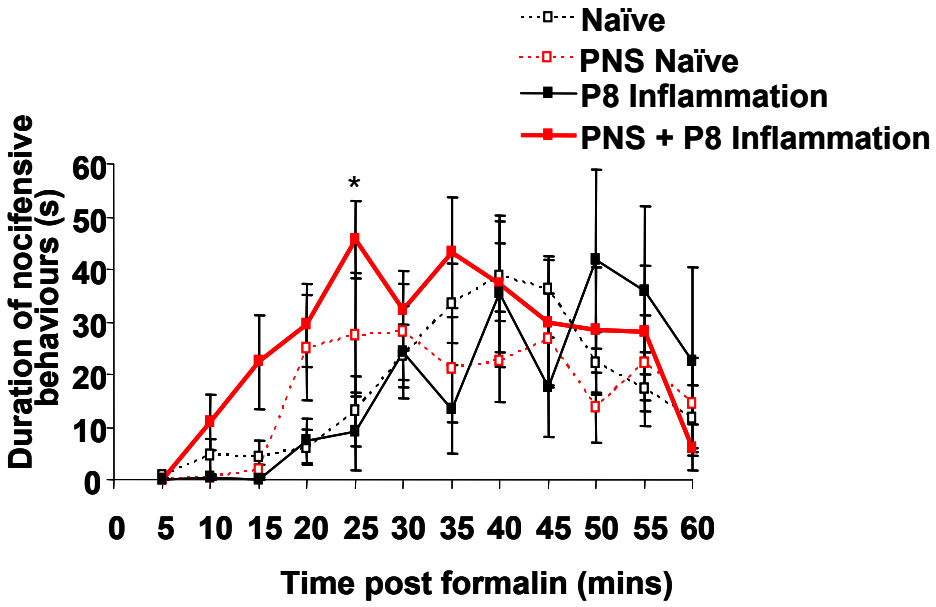


Figure 5.4. Adult male response to a formalin challenge in prenatally stressed animals (PNS, red) compared to non-stressed animals (black) following recovery from P8 inflammation (solid lines, n=3-5) or in uninjured (naïve) animals (dotted line n=6-10). Mean±SEM is plotted in seconds (s) for cumulative amount of nocifensive behaviour recorded per minute for each time point post-formalin injection. (*$p<0.05$, two way ANOVA with Bonferroni post test, PNS + P8 inflammation is different to naïve).

5.2.3 Prenatal stress reduces birth weight and enhances stress-induced HPA-axis responsiveness

Prenatally stressed male and female rats were found to have a significantly lower birth weight (P1) compared to non-stressed controls (Figure 5.5). Prenatally stressed females (n=32) and males (n=36) were significantly (p=0.002, and 0.005 respectively) lighter than controls (n=36 and n=30 respectively) of the same age (t-test). Additionally, prenatally stressed adult animals displayed higher stress-induced levels of plasma corticosterone (Figure 5.6). The formalin test that we use to assess the magnitude of nocifensive responsiveness also acts as a stressor as it is known to induce ACTH and corticosterone secretion (Taylor et al., 1998). Formalin significantly increased plasma corticosterone when compared to saline injection in

prenatally stressed adult males (t-test, p=0.00015) and importantly there was a significantly higher level of plasma corticosterone in response to formalin injection in prenatally stressed males compared to non-stressed animals (p=0.047). Adult female prenatally stressed animals also had higher levels of corticosterone in response to formalin compared to saline, although this did not reach statistical significance (p=0.082).

We also attempted to measured plasma ACTH in the same samples, (taken 60 minutes after formalin or saline injection) although these data were compromised due to lysed red blood cells (data not shown).

In addition to physiological assessments of possible programming in response to prenatal stress, we also carried out some pilot evaluations of affective behaviours using the EPM and open field tests in both pre-weanling and adult animals in response to early life (P8) or adult injury (nerve injury or inflammation). In these tests a reduced latency to enter, or reduced time spent in the open arms of the EPM or the exposed centre-square of the open field test reflects increased anxiety. However we did not observe any discernable differences in EPM performance between any of the groups, although numbers for each group were relatively small. We have however revealed a significant reduction (t-test, $p<0.05$) in the time spent grooming in the open-field test (Figure 5.7) in adult male prenatally stressed animals compared to non-stressed controls following recovery (2 months) from adult inflammation (n=4) or nerve injury (n=4), with a trend observed in naive (non-injured) prenatally stressed animals compared to naive controls (n=3).

Figure 5.5

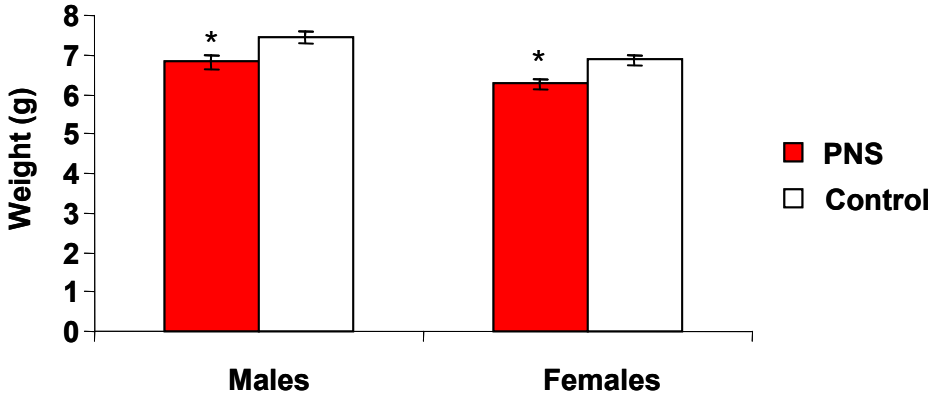


Figure 5.5. Effect of prenatal stress on birth weight (P1) of male (n=30-36) and female (n=32-36) offspring (*$p<0.05$, t-test compared to corresponding controls).

Figure 5.6

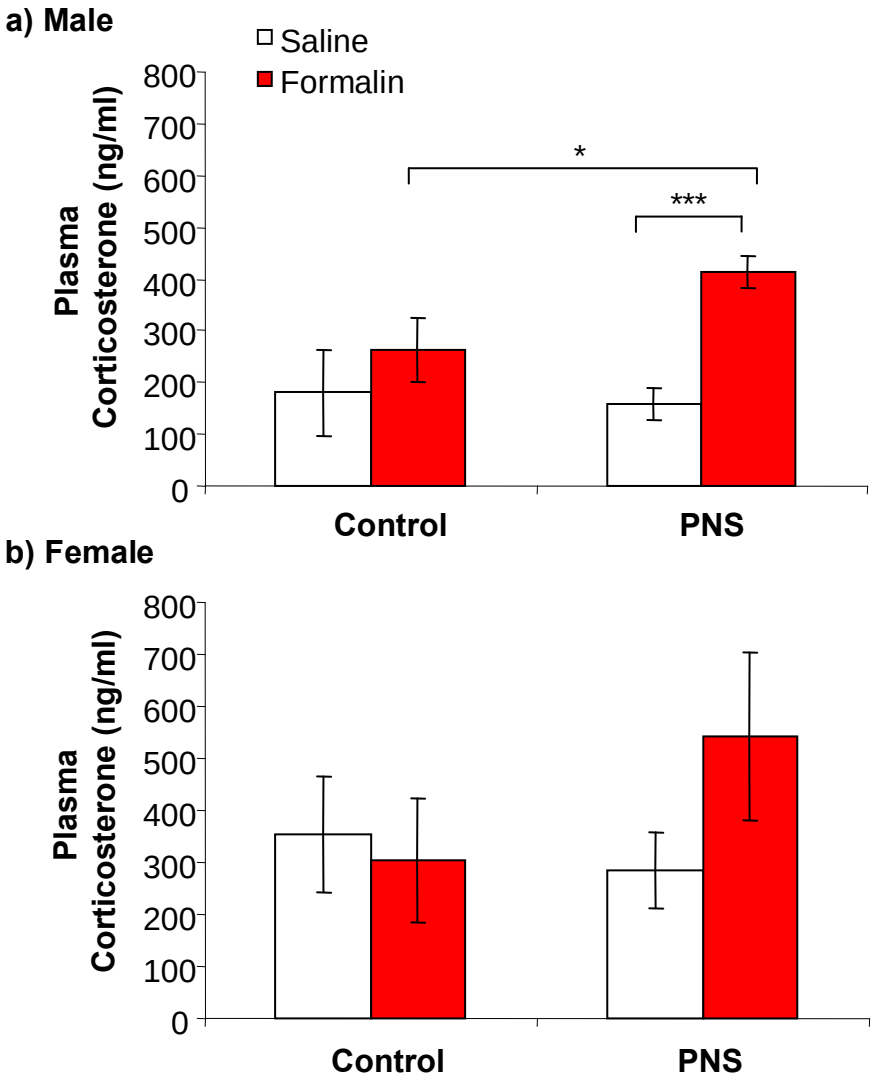


Figure 5.6. Plasma corticosterone concentrations (ng/ml, mean±SEM) in a) males (n=4-6) and b) females (n=4-5) following intraplantar injection of saline (□) or formalin (■) (*p<0.05, ***p<0.001, t-test).

Figure 5.7

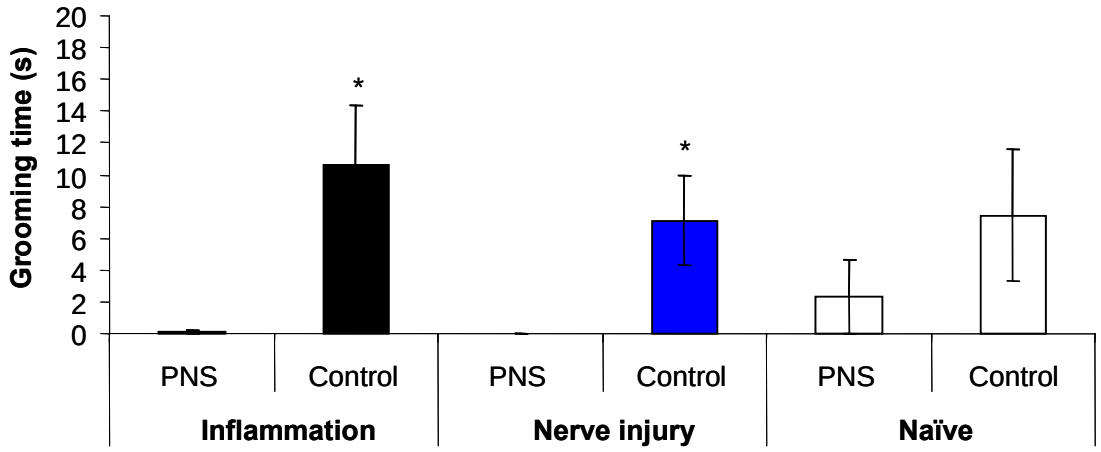


Figure 5.7. Time spent grooming during the open field test in prenatally stressed (PNS) or control males following recovery from adult inflammation (■ n=4), nerve injury (■ n=4), or naïve animals (□ n=3). (*p<0.05, t-test. PNS compared to control)

5.3 Discussion

5.3.1 Prenatal stress does not affect mechanical allodynia in response to early life or adult injury

Our data show that prenatal stress does not alter the mechanical allodynia observed following nerve injury or inflammation carried out at P8 (Figure 5.2). Similarly, there is little effect of prenatal stress on the nociceptive responses of adult-injured animals, although we did observe an increase in mechanical allodynia at day 6 after adult inflammation in prenatally stressed animals (Figure 5.1). Prenatal stress has been shown to alter immune function in offspring, although the effects seem to be entirely dependent on the type of maternal stress, and timing after birth when immune measures are taken (Merlot et al., 2008). Furthermore a recent study has shown that altered immune function is observed in offspring following maternal restraint stress towards the end of gestation, with no effects observed in juveniles (7 weeks old) but enhanced levels of pro-inflammatory cytokines by 6 months of age (Vanbesien-Mailliot et al., 2007). These data suggests that altered immune function could at least in part be responsible for the enhanced pain behaviour in response to inflammatory stimuli in prenatally stressed adult animals. Although we have not investigated this with direct measures of immune function, pro-inflammatory cytokines are known to be involved in chronic pain states (Sommer and Kress, 2004), so it is possible that increased circulating levels of pro-inflammatory cytokines in prenatally stressed animals may be involved in the enhanced sensitisation to mechanical stimuli in adult inflamed animals (Figure 5.1). Additionally, adult nociceptive thresholds have been shown to increase in animals exposed to prenatal stress during the last week of gestation, although this effect was reversed with repeated postnatal handling (Sternberg and Ridgway, 2003). This is an important observation, as in our study, adult injured animals remained with the mother until weaning, and so were not handled in the early postnatal period, whereas neonatally injured animals were repeatedly removed from the home cage for surgery and also for behaviour testing and so this raises the possibility that maternal behaviour following gestational stress and/or in response to these interventions may affect the

observed nociceptive responses. However it should be noted that we did not observe any obvious differences in maternal behaviour in the present study and other studies have reported that behaviour does not change in response to neonatal injury (LaPrairie and Murphy, 2007). Our work did not directly compare the effects of maternal care in mothers following gestational stress compared to non-stressed mothers, which has been reported to be altered in other studies (Baker et al., 2008), and therefore to address this, an interesting study might have been to cross-foster prenatally stressed and non-stressed offspring after birth to assess adult nociceptive behaviour following injury, to elucidate the importance of maternal behaviour on these thresholds. Furthermore, as we have only assessed mechanical allodynia as a measure of sensitisation in prenatally stressed versus non-stressed injured animals, it is possible that we may have missed differences which may have occurred in other modalities and so it might have been useful to have carried out tests using thermal stimuli for example.

5.3.2 Prenatal stress together with early life injury enhances nocifensive behaviour in response to formalin in later life

Our data show an enhanced nocifensive response to formalin in prenatally stressed and P8 inflamed animals in the early part of the second phase compared to naive controls (Figure 5.4). This effect occurs at around the same time as the interphase (between first and second phase) usually occurs. The interphase of the formalin response is thought to be dependent on active inhibitory pathways in response to afferent activity from the first phase (Henry et al., 1999; Gaumond et al., 2007). Furthermore, females and males differ in their nocifensive responses to formalin, and in particular, the interphase has been shown to be less suppressed in females compared to males, which is thought to be a result of two different inhibitory mechanisms; in females there appears to be an opioidergic involvement, believed to be related to sex hormones, whereas in males, GABA is thought to be the main transmitter involved (Kaneko and Hammond, 1997; Aloisi and Ceccarelli, 2000; Gaumond et al., 2007). Our results show that male prenatally stressed offspring have a leftward shifted time course in response to formalin, and therefore possibly less

inhibition of nocifensive behaviour in the "interphase" compared to non-stressed controls, although the effect is only significant in the prenatally stressed and postnatally injured group compared to naïve controls. Demasculinisation has been shown to occur in prenatally stressed male offspring (Weinstock, 2001), and it is possible that this may help to explain the leftward, and more female-like, shift in the time course of nocifensive behaviour in response to formalin, which might suggest an involvement of opioids in the interphase as opposed to testosterone-mediated mechanisms (Ceccarelli et al., 2003) in male prenatally stressed, postnatally injured males. Additionally there is evidence to suggest that descending oxytocin pathways from the PVN, part of the HPA axis, to the spinal cord are important in mediating descending inhibition, and have been suggested as a possible pathway involved in stress-induced analgesia (Robinson et al., 2002). Activation of the PVN in response to stress in known to result in release of oxytocin (Nishioka et al., 1998). Furthermore, oxytocin has been reported to be released in the spinal cord in response to stress (Miaskowski et al., 1988). As nociception is not altered in mice lacking oxytocin, it is thought that this pathway does not tonically regulate pain responses and is specifically activated and alters nociceptive thresholds in response to stress (Robinson et al., 2002). Furthermore, the spinal cord does not produce oxytocin, but expresses the oxytocin receptor (Reiter et al., 1994), so any influence of oxytocin might be expected to reflect supraspinal control. These pathways are thought to specifically inhibit activation of WDR neurons, which form part of the spinothalamic tract, by presynaptically inhibiting the nociceptive Aδ and C-fibres contacting these neurons (Rojas-Piloni et al., 2008). The inhibitory capability of this pathway is thought to involve recruitment of GABAergic interneurons, and also a possible opioid involvement (Breton et al., 2008; Condes-Lara et al., 2009).

Studies have shown that there is a reduction in oxytocin mRNA in the PVN in prenatally stressed animals (Lee et al., 2007) which may suggest a reduction in endogenous pain control via the PVN-spinal pathway. However this does not explain the lack of any altered responses to formalin in prenatally stressed and adult injured animals (Figure 5.3) and so it is likely that early postnatal pain is also an important factor in the "programming" of the formalin response following prenatal stress and P8 inflammation. Interestingly, studies have shown that early immune challenges (at

P3 and P5) are able to alter the stress responsiveness of the adult which is thought to be due to alterations to negative feedback control of ACTH by glucocorticoids (Shanks et al., 1995). In light of these studies and also now with our current data, it is becoming increasingly more apparent that both adverse pre and postnatal events can induce long-lasting changes in physiological responses to stress and pain in later life.

5.3.3 Prenatal social stress reduces birth weight and increases stress-induced corticosterone secretion

We have used a model of prenatal social stress that has been used by other groups and has shown robust changes in HPA axis responsiveness (Neumann et al., 2001). Although this social stress model is far more ethologically relevant, it is quite labour-intensive and wasteful in the numbers of animals needed (lactators and their pups) to generate experimental litters. For these reasons our experiments were designed in order to gain as much information from any one set of animals as possible, without compromising other important assessments. Unfortunately this means that robust assessment of HPA axis responsiveness has not been possible, and our measurements include those at the beginning and end of the life of the animals so as not to interfere with assessments of pain behaviour. We can never be sure how much offspring have been directly affected by maternal stress prenatally, as it is impossible to measure changes in fetal corticosterone *in utero*. However, the measures that we have been able to obtain indicate that a prenatal stress phenotype is present in the offspring from our socially stressed animals. These measures include low birth weight in prenatally stressed males and females compared to non-stressed controls (Figure 5.5), and an increased stress-induced corticosterone response (Figure 5.6) both of which are indicators of a prenatal stress phenotype, thought to occur in response to elevated maternal corticosterone, in agreement with other studies (Barbazanges et al., 1996; Welberg et al., 2000; Neumann et al., 2001).

Measures of affective behaviour (EPM and open field) were also taken to establish a prenatal stress phenotype, to compare our studies to those which report behaviours thought to represent increased anxiety in prenatally stressed animals (Weinstock, 2001; Neumann et al., 2001; Weinstock, 2008). In the present experiments we did

not observe any significant changes in classic signs of anxiety behaviour on the EPM or open field between prenatally stressed and non-stressed animals which may be due to small group sizes. However we did observe a decreased amount of time spent grooming during the open field test in prenatally stressed animals compared to non-stressed controls (Figure 5.7). This observation was only significantly different between prenatally stressed animals compared with non stressed, adult injured animals upon recovery from sensitisation, although there was also a trend in prenatally stressed naives compared to naïve, non-stressed controls. It is unclear what changes in this kind of behaviour might mean, grooming behaviours are thought to be a sign of habituation to a particular environment (Brenes et al., 2009) and it is possible that this may infer some signs of anxiety in animals with reduced grooming behaviours, although this is only interpretation and further studies would need to be carried out to clarify this.

5.4 Conclusions

Although it can only be described as preliminary due to the low numbers in most of the experimental groups, the current data suggest that adverse pre and postnatal events can both contribute to the induction of long-lasting changes in physiological responses to stress and pain in later life. Importantly, it seems that responses to early life (P8) or adult inflammatory insults are altered in prenatally stressed animals and in particular, males seem to be adversely affected. Further robust studies are needed to fully ascertain the effects of prenatal stress on female pain processing. Notable alterations of nocifensive behaviour in prenatally stressed animals were produced in response to inflammatory insults, which are common in clinical and husbandry practices. It is therefore important that further study following the combination of these two adverse events is carried out in order to elucidate the mechanisms responsible with the hope of preventing lasting negative changes in nociceptive processing.

CHAPTER 6: SUMMARY DISCUSSION AND CONCLUSIONS

6.1 Summary of findings

The aim of this study was to introduce a novel model of chronic neuropathic and inflammatory pain designed to model the injuries which occur in clinical situations where nerve injury may be accompanied by inflammation as a result of post-surgical infection. Once established in the adult, this model was then investigated in neonatal animals. We have shown that in the adult this combined pain model results in robust mechanical allodynia and thermal hyperalgesia which outlasts that observed following the single component injuries of this model. Furthermore, spontaneous pain is reliably induced in the combined pain model and lasts for a number of days. As spontaneous pain is one of the most commonly reported symptoms in chronic pain patients, it is important that this behaviour becomes more widely studied and this model may help to facilitate such investigations.

The development of the pathways necessary to experience and respond to painful or stressful stimuli can be modified at various levels and at a number of critical windows. Our data suggest that certain types of neonatal injury are capable of causing long-lasting changes to nociceptive processing. Specifically, young animals do not seem to respond to an inflammatory insult in the same way as an adult in terms of the sensitisation induced and this is perhaps the reason why the novel combined pain model does not enhance sensitisation compared to nerve injury alone, as it does in the adult. However, we have not investigated acute responses to an inflammatory insult at a young age and this would be beneficial to better assess the capabilities of neonatal animals to respond to an inflammatory agents.

It is important to highlight that these studies suggest, for the first time that early-life nerve injury, within a very specific developmental window at P8 but not P10, seems to be able to induce lasting NMDA-dependent mechanical allodynia. This finding is in agreement with other studies which suggest that nerve injury cannot induce neuropathic pain in young rats at P10 (Howard et al., 2005), but suggests that the timing of injury is critical. Additionally, P8 nerve-injured animals display altered nocifensive responses following formalin administration, particularly in females, following recovery from the injury-induced sensitisation, which indicates long-term

alterations to nociceptive processing. As the formalin response is not altered in response to adult injuries, our data highlight the vulnerability of the immature nervous system to such injuries. Further investigations into the specific mechanisms responsible for these changes will provide a greater insight into the capabilities of the immature nervous system to respond to noxious insults and how such interactions may result in long-lasting consequences.

A further aim of this study was to investigate the effects that prenatal social stress may have on these pain models. Our data suggest that prenatal stress alters nociceptive responses to inflammatory insults, which are common in clinical and husbandry practices. Specifically, adult prenatally stressed animals display greater sensitisation to mechanical stimuli following inflammation compared to non-stressed controls. Additionally, prenatally stressed and P8 inflamed animals display enhanced nocifensive behaviour in response to formalin in later-life.

6.2 Criticisms and future directions

The main criticism of this study has been the relatively low numbers of animals used in behavioural assessments, particularly within the data described in Chapter 5, where the nature of the prenatal stress studies has meant a limited number of animals have been used; this data can therefore only be presented as preliminarily data as it is largely incomplete. The compromise of utilising animals to achieve a range of different measures, and perhaps being too optimistic in what we set out to achieve has meant that group sizes have suffered and it is possible that sample sizes have been too small to obtain a statistical significance where small effects exist with the population. Power analysis would have been a beneficial addition to the experimental design of these experiments so that a small number of measures could have been carried out confidently, to give reliable and reproducible outcomes.

A further criticism lies within the testing methods we have employed in this study in an attempt to quantify pain following differing insults. Such measures have largely relied on spinally-mediated reflex responses to von Frey filaments. The use of such measures allows for a relatively quick and easily quantifiable output, however, there are limitations with both the nature of this type of test and also of the testing equipment itself. Criticisms of the latter are largely due to the variability within the

filaments themselves, which have been shown to vary with humidity and also with repeated use and so require regular calibration (Moller et al., 1998). Furthermore, the discrete nature of the data obtained means that precise thresholds are rarely achievable and the graded nature of the upper range of the hairs is such that a one hair difference can actually translate to a difference of more than 10g. This means that it is impossible to dissect out subtle differences within this range and instead the next available threshold is recorded. It is entirely possible that some of the differences recorded between ipsilateral and contralateral paws are exaggerated from von Frey hairs in the upper range, this may be the case in some of the adult data (Chapters 3 and 5) where this range is often needed to evoke responses. The lower range hairs were required for assessments in young animals (Chapter 4) and so the incremental increase is lower within this range, although it is still possible that thresholds are overestimated, and as there are such small apparent differences between the injured and uninjured paws in young animals, it is important that these measures are as accurate as possible so as to avoid false positives. Electronic devices which provide continuous data and accurate measurements of applied force have been reported (Moller et al., 1998) and may allow for a much more reliable means of assessing mechanical allodynia in both young and adult animals. However, there has also been some suggestion (Vierck et al., 2008) that these reflex measures are not entirely clinically relevant and that pre-clinical pain assessment should attempt to incorporate tests with a cortical dependency to allow for better translational pain research. In addition to the testing methods employed in this study, the way in which the experiments are carried out could have been improved to achieve more robust and reliable data. As previously mentioned (Chapter 2, 2.4), it was difficult to be blinded during the behavioural assessments due to the visible changes induced in response to injury. However, the inclusion of sham controls may have helped to minimise experimenter bias as the gait and posture of the animal would also be altered in these animals as in nerve injured animals, similarly, saline injected controls would have been a better control than naïve animals for the inflammatory insults.

6.3 Conclusions

Our studies have identified new animal models of pain that may occur as a result of clinical and husbandry practices, which, with further investigation, may help to inform us of the potentially adverse consequences of these practices. These data suggest, in accordance with other studies (Mastorci et al., 2009) that prenatal stress does not dramatically change normal functioning, but given a further insult, either adult inflammation or P8 inflammation plus formalin in later life, prenatally stressed animals are more susceptible than non-stressed animals, and this is when their pathophysiology is revealed. Given the many challenges an organism is likely to face during its life time, it is important that we understand the long-term impact that adverse perinatal events can have, and importantly, how and when such pathologies are likely to manifest themselves and what effect this will have on the individuals quality of life. Ultimately, once the mechanisms of perinatal programming of stress and pain processing are fully elucidated, it would be beneficial to be able to intervene to prevent those changes which result in negative outcomes in later life, whether this be by prevention, in raising awareness to enable avoidance of the perinatal events responsible, or by way of a cure to reverse a particular pathology either in its dormant phase or shortly after it is revealed.

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APPENDIX: Publications arising from research

Hayley L Gooding, Andrew J Allchorne and Susan Fleetwood-Walker (2008). Long-Lasting Changes In Sensory Processing Following Peripheral Nerve Injury In The Neonate. IASP, Glasgow, UK.

Andrew J Allchorne, Hayley L Gooding, Susan Fleetwood-Walker (2008). A Comparison Of The Expression Of Neuronal Activating Transcription Factor-3 In The Neonate And The Adult Rat Following Peripheral Nerve Injury. IASP, Glasgow, UK.

Abstract #1 Poster Presentation

International Association for the Study of Pain, 12th World Congress on Pain, Glasgow, UK, August 2008

LONG-LASTING CHANGES IN SENSORY PROCESSING FOLLOWING PERIPHERAL NERVE INJURY IN THE NEONATE

Hayley L Gooding, Andrew J Allchorne and Susan Fleetwood-Walker. Centre for Neuroscience Research, University of Edinburgh, Edinburgh, UK.

Aim of Investigation: The plasticity of the adult nervous system allows the development of long-lasting sensitised states following nerve injury or inflammation. In the neonate however, the impact of similar injuries is unclear. We hypothesise that neonatal nerve injury or inflammation will result in long-lasting changes to sensory processing, as revealed by a subsequent noxious challenge.

Methods: Adult or Postnatal day 8 (P8) Sprague-Dawley rats were either given an intraplantar injection of Complete Freund's adjuvant (1:1 CFA: saline, 1µl/g body weight) or a chronic constriction injury (CCI). Surgery to produce CCI consisted of two loose chromic gut sutures (5/0: P8 4/0: adult) tied proximal to the sciatic nerve trifurcation. Mechanical thresholds of the plantar surface of the ipsilateral and contralateral hindpaw were measured using von Frey filaments. Following recovery from the initial insult (>6 weeks) animals were subjected to a formalin challenge (intraplantar injection of 4% formalin solution 40 µl) and nocifensive behaviours were recorded.

Results: An inflammatory or neuropathic injury in adults produces long-lasting mechanical allodynia ipsilateral to injury. The equivalent insults carried out in neonates revealed insignificant differences in sensory thresholds following neonatal inflammation and a short-lasting decrease in thresholds following CCI. Interestingly, in response to a formalin challenge, neither of the adult pain models differed from controls, nor did animals with an inflammatory injury at P8, whilst those with a

neuropathic insult at P8 displayed significantly greater nocifensive behaviour than controls.

Conclusions: Neonatal nerve injury at a critical developmental time point can result in long-lasting and maybe permanent changes to nociceptive processing and could have adverse consequences in both clinical and certain husbandry practices.

Acknowledgements: This work is supported by a grant from the BBSRC Animal Welfare Programme Award

Abstract #2 Poster Presentation

International Association for the Study of Pain, 12th World Congress on Pain, Glasgow, UK, August 2008

A COMPARISON OF THE EXPRESSION OF NEURONAL ACTIVATING TRANSCRIPTION FACTOR-3 IN THE NEONATE AND THE ADULT RAT FOLLOWING PERIPHERAL NERVE INJURY.

Andrew J Allchorne, Hayley L Gooding, Susan Fleetwood-Walker

Aim: Activating Transcription Factor-3 (ATF3) is induced in dorsal root ganglia (DRG) sensory neurons following chronic constriction nerve injury (CCI) and is accompanied by increased sensitivity in adult rats. Neonatal rats are known to respond differently to nerve injury, so to address this we compared the DRG expression of ATF3 following CCI in rats at postnatal day 8 (P8) and in adults.

Methods:CCI nerve injury was carried out in P8 and adult Sprague Dawley rats using 2 loose chromic gut sutures tied proximal to the trifurcation of the sciatic nerve (size 5/0 suture for P8; 4/0 for adult). Mechanical withdrawal thresholds of the plantar surface of both ipsilateral and contralateral hindpaws were then measured using von Frey filaments. In a separate group of rats immunostaining for ATF3, neurofilament 200 (NF200) and peripherin (A and C fibre markers respectively) was carried out in fixed lumbar (L4-6) DRG 10 days after nerve injury.

Results: Adult rats showed a robust and significant decrease in mechanical nociceptive thresholds ipsilateral to injury and the neonate also showed an ipsilateral reduction. Although ATF3 immunoreactivity was observed both in adult and neonatal DRG neurons 10 days after injury, a time point when both groups show mechanical sensitivity, interestingly the expression level in the neonate was 3 fold greater compared to the adult (28.9±2% vs 9.9±2.1%, respectively). Co-localisation of ATF3 with NF200 and peripherin showed that ATF3 is found in both A and C fibres of adults and neonates, however neonates showed higher levels of ATF3

expression: 22.6±3.3% vs 11.3±3.2% and 34.6±1.6% vs 12.2±2.3% NF200 and peripherin positive neurons in neonate compared to adult, respectively.

Conclusions: Following CCI nerve injury there is a greater upregulation of the injury marker ATF3 in neonatal DRG compared to the adult, despite both neonates and adults developing sensitisation. This highlights the complex differences in response of the neonatal versus the adult nervous system to injury.

Acknowledgements: This work is supported by a BBSRC Animal Welfare Programme Award